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Ederio Dino Bidoia
Renato Nallin Montagnolli
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Toxicity and Biodegradation Testing

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Toxicity and Biodegradation Testing

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Preface

This book is designed to present a broad compendium of biodegradation and toxicological research. The concept for this book is to provide academic and industry researchers with an introduction to the current state of biodegradation studies and toxicological assessment. Chapters provide both legacy and up-to-date approaches to practical methodologies throughout the book, which were successfully applied to address real issues. This book also provides an overview of the role and applications of analytical biodegradation quantification as it applies to the environmental sciences, particularly in the range of by-products that are usually linked to toxicology, and the test organisms most often used in toxicity testing.

While the book is primarily focused toward the environmental sciences researcher, the range of techniques demonstrated in the book also provides an introduction to biodegradation and toxicology methods for researchers outside of this field. Chapters deal with a critical discussion of laboratory scale experiments, as well as full scale in situ and ex situ apparatus, with each chapter containing both a discursive section along with a detailed methods section. The topics of the book include scientific and technical feasibility studies, contaminant impacts evaluation, study design and analytical techniques, key methodologies required to prepare the biodegradation and toxicology protocols, as well as the handling of microbial communities related to such processes.

This book has been designed to serve as a comprehensive biotechnology textbook. The authors thank all those who have contributed significantly in understanding the different aspects of environmental sciences and submitted their chapters. We hope that our book will prove of equally high value to advanced undergraduate and graduate students, research scholars, and professionals.

Rio Claro, São Paulo, Brazil *Ederio Dino Bidoia*
Renato Nallin Montagnolli

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Chapter 1

Biodegradation of Selected Endocrine Disrupting Compounds

Tomasz Grześkowiak, Beata Czarczyńska-Goślińska, and Agnieszka Zgoła-Grześkowiak

Abstract

Nowadays, many different chemicals exerting negative effects on both human and animal health are widely present in the environment. Compounds that interfere in the action of endocrine system due to their structural similarities to the real hormones called endocrine disrupting chemicals have received much attention because they are suspected to affect reproduction, development, metabolism of living organisms and even induce cancer. Moreover, the endocrine-related disorders are often passed down to the next generations and alter their disease susceptibility. This group of substances includes both naturally occurring chemicals (e.g., phytoestrogen—coumestrol) and synthetic compounds used in industrial processes, agriculture, and household products (e.g., polychlorinated biphenyls, polybrominated biphenyls, polycyclic aromatic hydrocarbons, some pesticides, components of plastics such as bisphenols and phthalates). Among these compounds there are some groups of chemicals still widely used and therefore constituting an important source of health hazards. The most important man-made endocrine disrupting compounds belong to three groups which met with great interest in last years, i.e., phthalates, bisphenols (mainly bisphenol A), and alkylphenols (used mainly as ethoxylates). Decades of their production and usage led to considerable contamination of the environment. They are found in water, air, soil, both animal and plant food. Therefore, growing number of studies are devoted to their degradation, biodegradation, and removal from the environment. Present studies on the biodegradation of phthalates, bisphenols, and alkylphenol derivatives aim mainly at testing of selected bacterial strains of different lineage including some *Bacillus* sp., *Gordonia* sp., *Pseudoxanthomonas* sp., *Sphingomonas* sp., and *Rhodococcus* sp. bacteria as well as other bacterial strains. Tests with fungi like *Aspergillus* sp. and *Polyporus* sp. or fungal enzymes like laccases are also carried out. Ultimately, understanding metabolic pathways of diverse species and genes involved in the biodegradation may help in constructing bacterial or fungal strains through usage of genetic engineering for effective removal of selected endocrine disrupting compounds. On the other hand, studies on removal of these contaminants from the environment were also undertaken. Biodegradation in natural waters, including seawater and in soil and sediments was tested to gain information on possibility of their removal from contaminated areas.

Key words Endocrine disrupting compounds, Bisphenols, Alkylphenols, Phthalates

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1 Endocrine Disruption

Contamination of the environment is a serious problem in many countries due to increased production and usage of many chemicals exerting a negative effect on both human and animal health. Some of these compounds interfere in the action of endocrine system due to their structural similarities to the real hormones. They enter cells and mimic natural hormones or block their activity (Fig. 1). These compounds are called Endocrine Disrupting Chemicals (EDCs) and they belong to the most widely determined substances. Their determination has received much attention because EDCs are suspected to affect reproduction, development, metabolism of living organisms and even induce cancer. EDCs can reduce sperm count

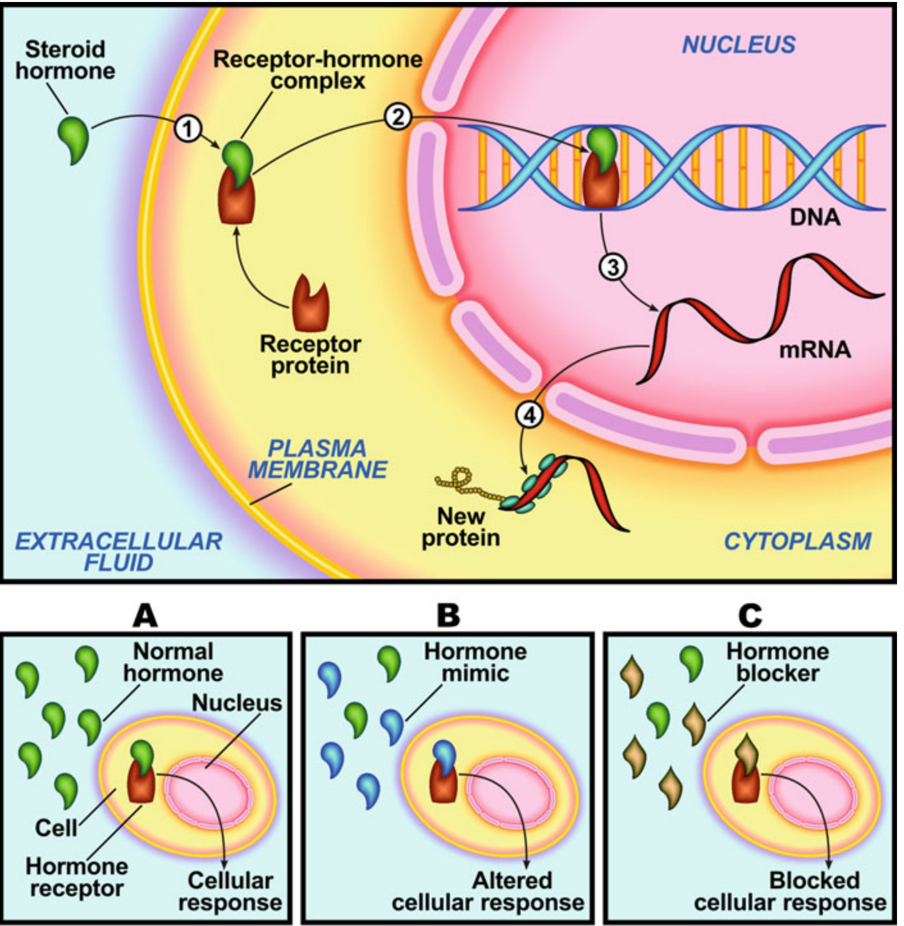

Fig. 1 A scheme of a hormone affecting its target cell. *1*—The hormone diffuses through the plasma membrane and binds a receptor. *2*—The receptor-hormone complex enters the nucleus and binds a specific DNA sequence. *3*—Binding initiates transcription of the gene to mRNA. *4*—mRNA directs synthesis of a protein. (**a**) Normal cellular response to a hormone. (**b**) Cellular response altered by an endocrine disrupting chemical. (**c**) Cellular response blocked by an endocrine disrupting chemical

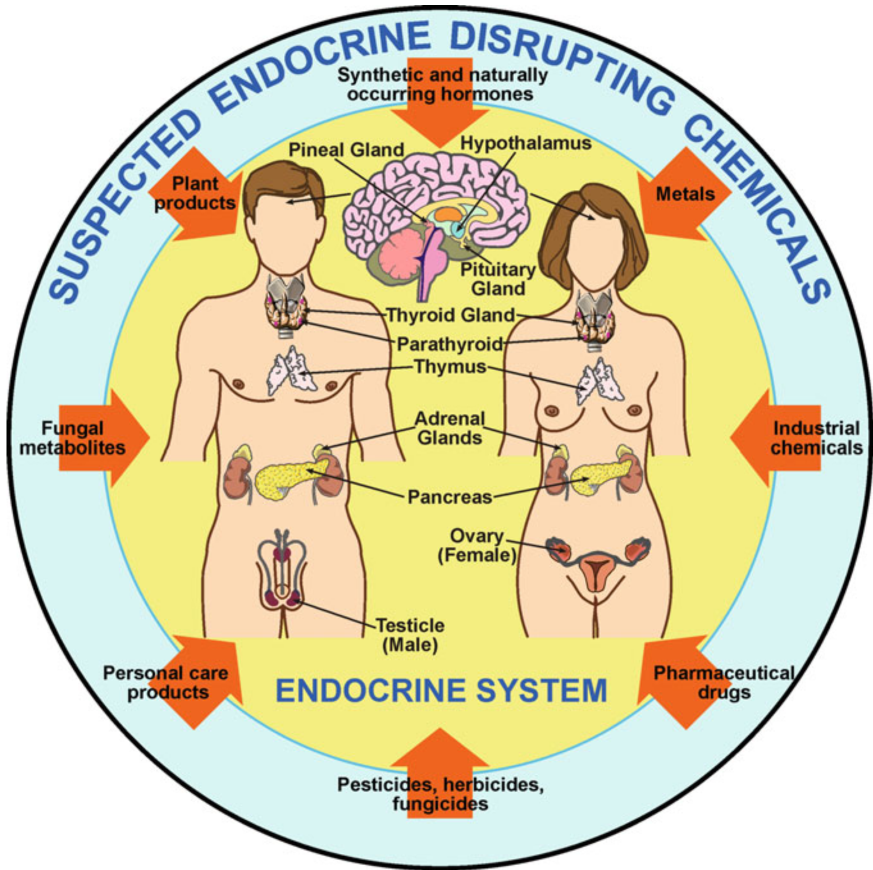

Fig. 2 A scheme of endocrine disrupting chemicals of different origin influencing the human endocrine system

and quality, increasing the number of testicular germ cells and inducing male breast cancer, cryptorchidism, hypospadias, miscarriages, disorders of the menstrual cycle, infertility [1]. Moreover, the endocrine-related disorders are often passed down to the next generations and alter their disease susceptibility. This group of substances includes both naturally occurring chemicals (e.g., phytoestrogen—coumestrol) and synthetic compounds used in industry and household processes such as polychlorinated biphenyls, polibrominated biphenyls, alkylphenols and their ethoxylates, dioxins, polycyclic aromatic hydrocarbons, dichlorodiphenyltrichloroethane, bisphenol A, and phthalates [2–4]. EDCs occur in both domestic and workplace circumstances. They are identified in water, air, food (in plants and animals), cosmetics, and pharmaceuticals (Fig. 2).

1.1 Endocrine Disrupting Properties of Bisphenol A

Bisphenol A (BPA) is frequently found in many consumer products such as plastic bags and containers, sports equipment, CDs, and DVDs. Because of its cross-linking properties in the manufacture of polycarbonate materials and epoxy resins, this compound has been

widely used in the production of plastics since 1957 [5, 6]. Therefore, leaching of BPA from polycarbonate plastics, baby bottles, dental sealants, epoxy resin linings for food and beverage containers, water pipes, and thermal papers makes it a ubiquitous contaminant of the environment [7, 8]. It can bioaccumulate both in ground water, wastewater, and air leading to continual exposure of humans and animals, whereas for most species food is the major route of exposure [9]. Unconjugated BPA has been repeatedly detected in human blood, breast milk, amniotic fluid, and placental tissue at ng/ml concentrations. The levels found in the blood of pregnant women, fetal blood, umbilical cords, placenta, and amniotic fluid are alarming because of the acute toxicity of the developing fetus to xenobiotics [10]. BPA may be linked with diabetes, obesity, cancer, cardiovascular and kidney diseases [11]. However, it is an analogue of estrogen and is first suspected to affect reproduction, development, metabolism of living organisms. Prenatal exposure to BPA is associated with the emergence of endometriosis [12]. An et al. studied the effects of BPA and 4-*tert*-octylphenol (OP) on uterine contractions in immature rats by analyzing the expression and localization of contraction-associated proteins, and contractility of rat uterus. OP and BPA increased oxytocin-related pathway, reduced the prostaglandin-related signaling and the contractility, which indicated their interference with uterine contractility [13]. Biological impact of BPA over the long term was tested on the animal model *Caenorhabditis elegans*. Their exposure to BPA (0.0001–10 μM) from L4 larvae to day-10 adult resulted in significant adverse effects and led to strong stress responses in vivo. In comparison to the short-term toxicity evaluation, chronic exposure to BPA caused a more obvious stress response at the same concentration, probably due to cumulative toxic effects [14]. Other studies on the effects of exposure to BPA in adult male Wistar rats revealed the reproductive toxicity of BPA at dosages considered nontoxic (5 or 25 mg/kg/day). Research outcomes demonstrated that BPA at safe dosages compromises the spermatozoa and disrupts the hypothalamic–pituitary–gonadal axis, causing a state of hypogonadotropic hypogonadism [15].

Also the impact of BPA on human fetal lung fibroblasts was meticulously reviewed. Majority of studies have been conducted on mice pups, and their results showed the adverse effects of BPA on normal fetal lung and airway development caused by high BPA concentrations in maternal food. Although the results of research are sometimes conflicting or difficult to correlate from animal models to humans, BPA contributes to the prenatal and postnatal development of asthma [11].

1.2 Endocrine Disrupting Properties of Alkylphenols

Another group of persistent chemicals in the environment are alkylphenols (APs) and their derivatives. These pollutants are both of natural (e.g., crude oils) and anthropogenic origin (e.g., substances widely applied in industry, agriculture, and households) [16]. As a result of the degradation of alkylphenolethoxylates (APEs) in wastewater treatment plants or in the environment, ubiquitous short-chain APEs and APs such as *p*-nonylphenol (NP), *p*-octylphenol (OP), and AP mono- to triethoxylates (APE1, APE2, and APE3) have been widely identified [17, 18]. Intensive research focuses on the toxicity of the derivatives of alkylphenols, especially on aquatic organisms. They affect endocrine system in fish and mammals and act both as agonists and antagonists of endogenous estrogens depending on concentration. Moreover, they have carcinogenic, hepatotoxic, genotoxic potential and impact on some behavioral modulators [19]. Estrogenic activity of *p*-alkylphenols was first proved by Dodds and Lawson in 1938 [20]. These observations were confirmed by Mueller and Kim [21] who studied the ability of various alkylphenols to displace the prebound estradiol from estrogen receptor of uterine cytosols and also to avoid binding estradiol to the receptors. The ubiquitous exposure of aquatic organisms to synthetic estrogens became evident. Tabata et al. studied the influence of NP, BPA, and 17β-estradiol (E2) on mature male medaka. Exposure to all these substances resulted in induction of female-specific proteins in the following order E2 > NP > BPA. Abnormal female-like anal fin was noticed in male exposed to 100 ppb of NP. Although the concentrations of both NP and BPA in water were lower than the LC_{50} values for these substances, they have still posed a threat to aquatic organisms due to their estrogenic potential [22]. Meier et al. studied the effects of APs on the reproductive potential of first-time spawning Atlantic cod (*Gadus morhua*). In male fish AP-exposure reduced 11-keto-testosterone concentrations, and slightly induced levels of a typically female protein—vitellogenin. Additionally, they observed the impairment of testicular development with the increased amount of spermatogonia and a decreased amount of spermatozoa present [23]. In another study, Meier et al. investigated the impact of large amounts of APs included in the produced water released in large quantities into the North Sea on Atlantic cod (*Gadusmorhua*). The results showed that fish exposure to low concentrations of APs could affect the timing of the onset of puberty [24]. Studies of Feng at al. revealed the effects of the endocrine disruption of NP on the sperm dynamic parameters of amphibian *Bufo raddei* during the period of ejaculation and insemination [25]. Furthermore, male rats were treated with varying concentrations of NP (0, 5, 20, and 60 mg/kg/2 days), where 60 mg/kg NP increased occurrence of sperm deformities, impaired epididymal sperm function, fertilizing capacity and caused seminiferous tubule degeneration [26].

1.3 Endocrine Disrupting Properties of Phthalates

Phthalates are esters of phthalic acid commonly used as plasticizing agents to improve flexibility and durability of plastics. They are applied in many branches of industry and are found from pharmaceuticals, cosmetics, automotive articles to food and childcare products. To the most popular phthalates belong di-2-ethyl-hexyl phthalate (DEHP), di-isononyl phthalate (DINP), di-isodecyl phthalate (DIDP), and benzyl-butyl phthalate (BBP). Recently, harmful effects of phthalates on multiple biochemical processes in humans and wildlife have received much attention. They interfere in the reproductive system of animals and similar disorders can be expected in humans. According to research conducted by Axelsson et al. DEHP metabolite levels were negatively associated with progressive sperm motility and men in the highest quartile of the DEHP metabolite monoethylhexyl phthalate were associated with a lower proportion of mature spermatozoa than men in the lowest quartile [27]. Additionally, Cai et al. performed a meta-analysis based on 14 studies and also confirmed that specific phthalate exposures can actually be linked with the incidence of reduced human semen quality [28]. The Danish scientists found relation between delayed pubarche, and high phthalate excretion in urine of healthy girls, which may indicate anti-androgenic potential of phthalates [29].

Some phthalates are probably able to interfere in thyroid function. Studies performed on serum and urine of Taiwanese pregnant women confirmed the relation between exposure to di-*n*-butyl phthalate (DBP) and impact on thyroid activity leading to maternal hypothyroidism and next adverse effects in the fetus [30].

Phthalates may be related to cancer in humans and suspected to increase the risk of asthma and allergies. López-Carrillo et al. testing urinary concentrations of nine phthalate metabolites in Mexican women found out that diethyl phthalate, the parent compound of monoethyl phthalate, may increase risk of breast cancer incidence [31]. Next, the Chinese researchers investigated the association between DEHP and asthma predominance in mice and confirmed the hypothesis that DEHP may promote or aggravate allergic asthma [32]. Furthermore, Taiwanese scientists reviewing many epidemiological studies confirmed that phthalates are believed to affect directly airway epithelial cells and contribute to their remodeling, which makes them important environment pathogens of asthma [33].

Last but not least, phthalates may play even a key role in the pathogenesis of autism [6, 34, 35]. Studies performed by Testa et al. confirmed the assumption of correlations between phthalates exposure and autism spectrum disorders (ASDs) in children. The urine concentrations of the primary and secondary metabolites of DEHP were measured and in ASD patients significant increase in mono-(2-ethyl-5-hydroxyhexyl) 1,2-benzenedicarboxylate (5-OH-MEHP) and mono-(2-ethyl-5-oxohexyl) 1,2-benzenedicarboxylate (5-oxo-

MEHP) urinary concentrations was found as well as an evident positive correlation between these two oxidized metabolites was revealed. The increased levels of fully oxidized form 5-oxo-MEHP are characteristic of patients with ASDs showing 91.1% specificity in identifying these patients [36].

2 Biodegradation of Endocrine Disrupting Compounds

Biodegradation testing of chemicals (especially EDCs) is important because information on their degradability may be used for hazard or risk assessment. This assessment is generally based on data obtained from ready biodegradability tests but results from simulation tests, inherent biodegradability tests, and anaerobic biodegradability tests can also be taken into account [37].

In the ready biodegradability tests relatively high concentrations of the test substance are used (up to 100 mg/L) and biodegradation is measured using nonspecific parameters like dissolved organic carbon (DOC), biochemical oxygen demand, or CO_2 production. A non-preadapted inoculum from domestic sewage, activated sludge, or secondary effluent (from a sewage treatment plant) is usually applied in these tests. Depending on parameter used for assessment biodegradation assessment between 60% (for theoretical oxygen demand and CO_2 production) and 70% (for DOC removal) must be reached during a 10-day window in a 28-day test. The remaining 30–40% of the test substance is assumed to be assimilated by the biomass or present as products of biosynthesis. A chemical attaining the pass level in these tests is classified as readily biodegradable and a positive result can be considered indicative of rapid and ultimate degradation in most environments [37].

If a negative result in a ready biodegradability test is obtained, biodegradation can be studied in simulation tests. These tests simulate degradation in a specific environment like soil, sediment, water or in a sewage treatment plant. Higher concentrations of the test substance are usually applied for tests simulating sewage treatment plants while being lower for this simulating degradation in the environment. Higher concentrations of the tested compounds can also be used to identify transformation products. The results of the simulation tests may include disappearance of the parent compound by chemical analysis (which does not mean mineralization), degradation half-life, pathways of transformation, etc. [37].

The inherent biodegradability tests have been designed to assess whether there is any potential for aerobic biodegradation of the tested compound. Inherent biodegradability can be measured by a compound-specific determination (for primary biodegradation) or by a nonspecific analysis (for ultimate biodegradation). Biodegradation above 20% of theoretical may be considered

evidence of inherent primary biodegradability and results above 70% may be regarded as evidence of inherent ultimate biodegradability [37].

2.1 Biodegradation of Bisphenols

Bisphenol A is widely known because of its endocrine disrupting properties. A number of studies showed its widespread occurrence in plastic products including toys, food containers, personal care product containers etc. Thus, BPA was found to be a real threat to people. Moreover, it was also found in the environment posing risk to both humans and animals. A number of studies on its endocrine properties and widespread occurrence led to its partial replacement with other bisphenols including bisphenol F (bis(4-hydroxyphenyl) methane), bisphenol S (bis(4-hydroxyphenyl)sulfone), and other types. As has been lately found these compounds can also be classified as endocrine disrupting compounds [38–41]. Nevertheless, still the most widely published studies relate to BPA including research on its biodegradation. Some studies presented biodegradation of bisphenols in environmental water and soils [42–44]. These studies are difficult in interpretation because of very different types of environmental matrices and therefore a number of various bacterial strains present. However, it must be underlined that high and relatively fast biodegradation of BPA was noted in river waters. Dorn et al. tested degradation using natural waters from the vicinity of a chemical plant producing BPA as well as effluent from a wastewater treatment plant. Greater than 90% degradation was confirmed in all treatments after 5 days [42]. Wu et al. tested biodegradation in river water and distilled water (pure or spiked with heavy metals or humic acid). About 80–90% degradation of BPA was achieved in 4 days [43]. Nevertheless, it is known that even 90% degradation of hydrophobic compounds leads to their accumulation in sediments or soil. Therefore, studies in this environment were also undertaken. Langdon et al. experimented with biodegradation of BPA in biosolids-amended soil testing both indigenous and spiked compound. It was found that spiked BPA was degraded approximately five times faster than the indigenous compound [44]. Moreover, there was serious amount of recalcitrant BPA found in the tests with indigenous compound corresponding to 23–33% of the initial concentration. As it was suggested problems with incomplete degradation may be due to limited oxygen availability within soil aggregates or non-reversible sorption of the compound. Nevertheless, the most important conclusion to be drawn from this study is that the use of spike degradation experiments may not provide an accurate assessment of the persistence and biodegradation of organic compounds [44].

Although biodegradation of BPA described above gives some important information, a comparative study on different bisphenols by Danzl et al. could also supply even more interesting data. In the study the authors compared degradation of BPA, bisphenol F

(BPF) and bisphenol S (BFS) in seawater using the sea die-away and Total Organic Carbon (TOC) Handai tests [45]. No degradation of BPS was found in a number of tests with seawater taken from two sites in three different months. On the contrary, both BPA and BPF almost completely degraded in most of the sea die-away tests in 2 weeks. Much lower degradation efficiency was noted in the TOC Handai test suggesting that complete degradation of these two bisphenols was not completed. Considerably better degradation of BPF than BPA found by the authors was attributed to their chemical structures, i.e., to easier attack on the hydrogen atom in the methylene ring-linking group of BPF than on the ring-linking fragment of BPA [45]. In this context, the sulfonyl group of BPS was the main reason for the lack of biodegradation of this compound.

Many latest publications on biodegradation of bisphenols included studies on bacterial strains isolated from activated sludge [46], soil [47], sediment [48], water [49, 50], and other sources [51]. A number of different bacterial strains were isolated in these studies showing that biodegradation of BPA (and some other bisphenols) can be easily achieved. Lobos et al. isolated from a sludge (but not fully identified) MV1 strain degrading BPA and other bisphenols including: bis(4-hydroxyphenyl)methane, bis(4-hydroxyphenyl) ethane, 2,2-bis(4-hydroxy-3-methylphenyl)propane, 2,2-bis(4-methoxyphenyl)propane, 2,2-bis(4-hydroxyphenyl)butane, 1,1-bis(4-hydroxyphenyl)cyclopentane, 3,3-bis(4-hydroxyphenyl)pentane, and *trans*-4,4′-dihydroxystilbene [46]. For BPA two biodegradation pathways were presented. In the major route 4-hydroxyacetophenone and 4-hydroxybenzoic acid were formed while in the minor route the products included 2,2-bis(4-hydroxyphenyl)-1-propanol and 2,3-bis(4-hydroxyphenyl)-1,2-propanediol [46]. A study on the formation of biodegradation products was continued by the authors who supplemented the biodegradation scheme with a few more biodegradation products (Fig. 3) [52].

Matsumura et al. collected over a hundred of soil samples and after addition of BPA identified 85 soil samples capable of its degradation [47]. The authors isolated and identified 26 different bacterial strains from these soils which were used in further biodegradation tests. Depending on culture medium and strain biodegradation of BPA was achieved in 12–60 h. During these tests different metabolites were identified (depending on strains used in the test) including 4,4′-dihydroxy-α-methylstilbene, 1,2-bis(4-hydroxyphenyl)-2-propanol, 2,2-bis(4-hydroxyphenyl)-1-propanol, and 4-hydroxyacetophenone [47].

Peng et al. isolated bacterial strains from river sediment [48]. All four strains isolated from the sample belong to *Pseudomonas knackmussii* spp. and degraded BPA from 7 to 9 days. However, during the test with a mixture of these strains biodegradation

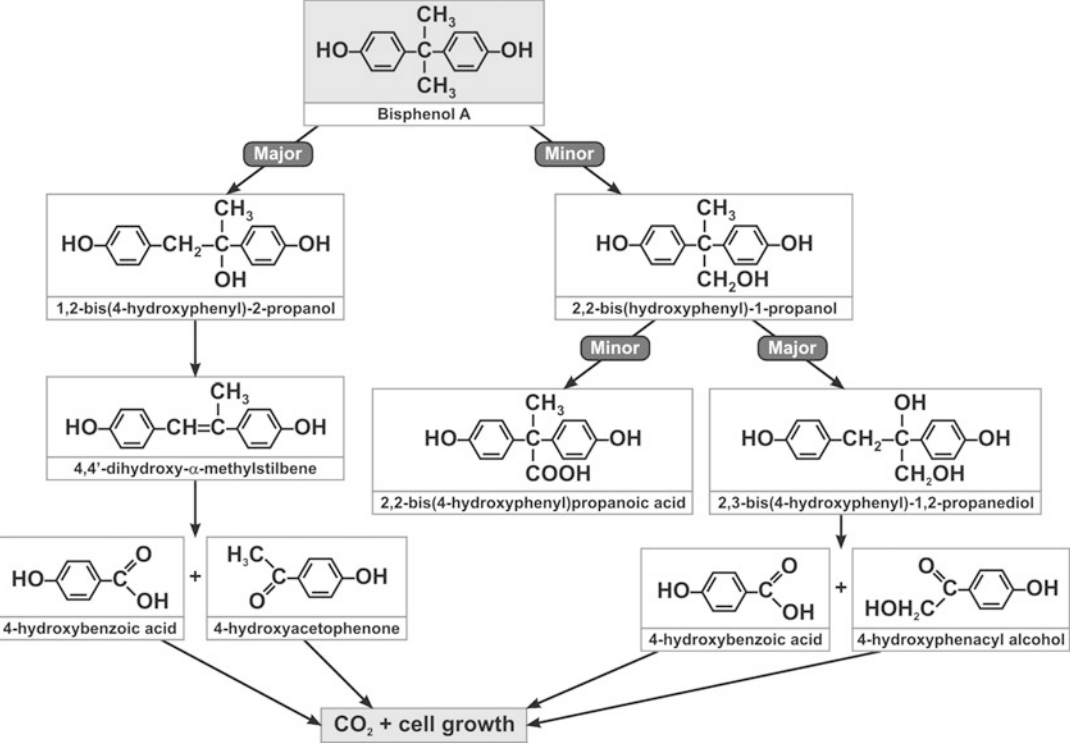

Fig. 3 A biodegradation scheme of bisphenol A

was finished in merely 6 days which points to their close cooperation. Therefore, microbial diversity in the consortium should be considered during remediation. Among metabolites six compounds were identified: 2,2-bis(4-hydroxyphenyl)-1-propanol, 1,2-bis(4-hydroxyphenyl)-2-propanol, carbocationicisopropylphenol, 4-isopropenylphenol, 4,4′-dihydroxy-α-methylstilbene, and 2,2-bis(4-hydroxyphenyl)propanoic acid [48]. Four of these compounds were also identified by Matsumura et al. [47] pointing to existence of some similar biodegradation pathways.

A very interesting study on a wide group of bisphenols and structurally related compounds was presented by Sakai et al. [49]. The authors isolated from seawater *Sphingomonas* sp. strain BP-7 degrading BPA. Depending on the medium they noticed no degradation or complete degradation. High degradation was possible only in the presence of peptone with or without additional nutrients (like beef extract, yeast extract, vitamins, or pyrroloquinolinequinone). For medium containing yeast extract and mineral salts addition of *Pseudomonas* sp. strain BP-14 (unable to degrade BPA on its own) considerably accelerated biodegradation process (from over 40 day to <10 days) [49] which confirms advantage of microbial diversity as it was reported by Peng et al. [48]. The isolated *Sphingomonas* sp. strain BP-7 was tested for biodegradation ability

of several other compounds and three of them stayed intact including BPF, BPS, and bis(4-hydroxyphenyl)sulfide [49]. Unfortunately, the first two of these compounds are widely used as substitutes for BPA.

Degradation of BPF was studied by Inoue et al. [50]. The authors isolated *Sphingobium yanoikuyae* strain FM-2 from river water. The strain pre-incubated on BPF was able to degrade this compound within 9 h. The proposed biodegradation pathway included oxidation of the methylene group leading to bis(4-hydroxyphenyl)methanol, 4,4′-dihydroxybenzophenone and further to 4-hydroxyphenyl 4-hydroxybenzoate, which was decomposed to 4-hydroxybenzoate and 1,4-hydroquinone [50].

A good source of bacteria degrading BPA was also found in petrochemical wastewater. Badiefar et al. isolated nine strains capable of its degradation and selected one for further studies [51]. It was identified as *Enterobacter gergoviae* strain BYK-7. The authors genetically enhanced its capabilities by introducing *bisdAB* operon. This operon encodes cytochrome P450 responsible for the degradation of BPA in the *Sphingomonas bisphenolicum* strain AO1. After the introduction of the *bisdAB* operon the modified *Enterobacter gergoviae* BYK-7 strain was tested for BPA degradation activity. Although its activity was lower after 8 h, considerable improvement was noted after 24 and 48 h. The modified strain was able to remove two times more BPA than the unmodified one [51].

The above-mentioned papers give rise to a statement that BPA can be easily degraded by many bacterial strains. However, it must be considered that some of the biodegradation products given above can accumulate in the environment and their endocrine disrupting properties are unknown. This topic was studied by Ike et al. who found that the biodegradation products from the major degradation route (4-hydroxyacetophenone, 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid) neither accumulate nor demonstrate estrogenic properties [53]. Two compounds from the minor route (2,3-bis(4-hydroxyphenyl)-1,2-propanediol and 4-hydroxyphenacyl alcohol) accumulated but collaterally these compounds were not tested for their estrogenic properties because they were unavailable. Taking into account that about 15% of initial TOC was still determined after almost 50 days of biodegradation [53], accumulation of these compounds can be a problem, especially 2,3-bis(4-hydroxyphenyl)-1,2-propanediol that is structurally similar to BPA.

Apart from studies on bacterial biodegradation of BPA, a relatively small number of papers on fungal degradation were also published. Different fungi were isolated and used for testing. Results included removal of BPA and identification of biodegradation products and analysis of estrogenic activity. Fouda et al. isolated 52 fungal species from mid-depth layers of six soil samples contaminated with phenolic compounds [54]. The isolates were

screened for biodegradation capability of BPA and only two isolates succeeded to grow on this compound, i.e., *Aspergillus terreus* and *Aspergillus flavus.* Shin et al. tested a number of white rot fungi strains for the biodegradation of BPA [55]. Initial test revealed that *Irpex lacteus* strain removed entire BPA in 12 h and *Trametes versicolor* strain removed 98.2%. What is more, *Irpex lacteus* strain degraded 99.4% of BPA in only 3 h. Nevertheless, it was also found that BPA was toxic to *Irpex lacteus* strain and (depending on its concentration) up to 73.6% of dry weight of mycelium decreased during 48 h of incubation in a medium containing this compound [55]. Lee et al. selected two strains *Stereum hirsutum* and *Heterobasidion insulare* by screening various white rot fungi [56]. Initial experiments revealed high resistance of these two strains to BPA. The fungal mycelium growth was slightly lower than in control samples up to 100 ppm of BPA, but further increase of concentration to 500 ppm stopped the growth. Test at 200 ppm showed only traces of BPA after 7 days and its complete degradation after 14 days. As it was shown the extracellular enzyme systems of both fungi were not strongly induced but a slight increase in laccase and manganese peroxidase activities was observed. There were only four metabolites found in the study (2-hydroxy-3-phenyl propanoic acid, 4-methoxyphenyl ethane, 2-phenyl acetic acid, and 2-hydroxy-2-phenyl acetic acid) and none of them was found in bacterial degradation of BPA. What is more important, a gradual reduction in estrogenic activity was also observed [56].

Studies on fungal biodegradation of bisphenols were supplemented with tests free from fungi but with fungal enzymes. Daâssi et al. [57] tested different fungal laccases from strains *Coriolopsis gallica* (BS54) [KJ412304], *Bjerkandera adusta* (11B) [KU904462], and *Trametes versicolor* (A3) [KU904463]. Laccases from *Bjerkandera adusta* and *Trametes versicolor* in the presence of mediator (1-hydroxybenzotriazole) eliminated BPA during 8 h and laccase from *Coriolopsis gallica* in 4 h. The biodegradation products differed when different laccases were used or when no mediator was used. For example, usage of the laccase from *Coriolopsis gallica* led to the formation of 3-hydroxybutyric acid and after addition of mediator tartaric acid and pyroglutamic acid was identified [57]. On the other hand, Uchida et al. reported formation of polymeric degradation product when using laccase from *Trametes villosa* [58].

A more practical approach was tested by Nicolucci et al. who examined biodegradation of bisphenols using immobilized laccase and tyrosinase [59]. The enzymes were immobilized on polyacrylonitrile beads and placed in a fluidized bed reactor for continuous removal of bisphenols. Immobilized laccase enabled removal of four tested bisphenols (BPA, bisphenol B (2,2-bis(4-hydroxyphenyl)butane), BPF and tetrachlorobisphenol A) in 90 min. Immobilized tyrosinase was slightly less efficient and removed more than 90% of bisphenols in 90 min. Moreover, stability of immobilized

laccase was much higher than that of the free laccase. When relative activity of the free laccase was reduced in 30 days to a few percent of the initial value, simultaneously tested relative activity of the immobilized laccase was only 10% lower than the initial value. This immobilized enzyme is also less sensitive to pH and temperature changes which gives high potential in wastewater treatment [59].

2.2 Biodegradation of Alkylphenols and Their Ethoxylates

Biodegradation of alkylphenolethoxylates was extensively studied beginning from the late twentieth century. A number of studies showed their relatively fast primary biodegradation in numerous tests [60–63]. During the tests both oxidative and non-oxidative biodegradation pathways were found. In both these pathways shortening of the ethoxy chain was found to lead to short-chain alkylphenolethoxylates in the non-oxidative route and their carboxylated derivatives in the oxidative route [60–62]. Both these types of biodegradation products as well as alkylphenol produced during further biodegradation were found to interfere with the endocrine system [64, 65]. Some studies suggested also direct formation of alkylphenols from their ethoxylates during the central scission process with simultaneous release of polyethylene glycols (Fig. 4) [66]. In other studies biodegradation products with

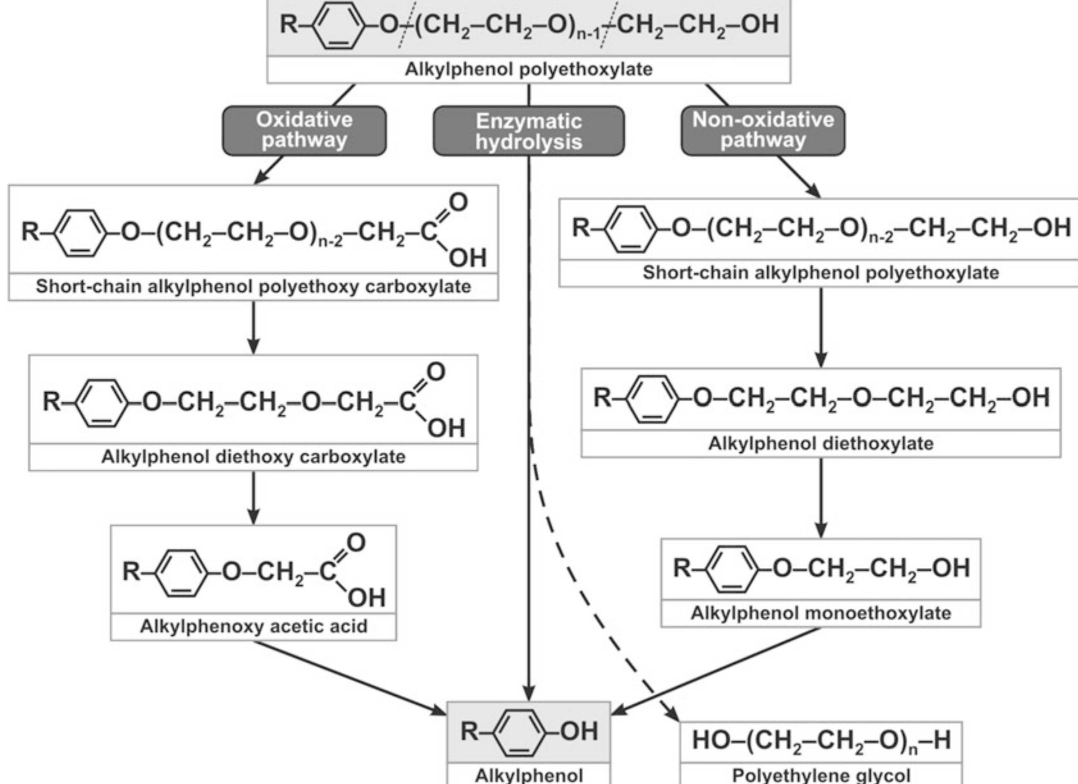

Fig. 4 A biodegradation scheme of alkylphenol ethoxylates

carboxy groups in the alkyl chain were also found [67, 68], but their endocrine disrupting properties were not evaluated due to their minor occurrence. Extensive studies showing high environmental impact of alkylhenols and their short-chain ethoxylates including studies on their presence in sewage effluents as well as on their accumulation in the environment [69–71] led to law regulations reducing usage of nonylphenol and its ethoxylates being the main thread due to their massive usage [72]. Furthermore, extensive studies on isolation of organisms capable of degrading alkylphenols and their ethoxylates were undertaken. A number of different bacteria were isolated from activated sludge [73, 74] or soil [75]. The isolated bacterial strains were capable of degrading alkylphenol ethoxylates. Nevertheless, accumulation of short-chain alkylphenol ethoxylates and their carboxylated derivatives still took place similarly to previous studies on mixed bacterial cultures.

Maki et al. isolated *Pseudomonas* sp. strain TR01 from activated sludge [73]. This strain enabled relatively fast biodegradation of nonylphenol ethoxylates with substantial shortening of ethoxy chain within a few days. However, the strain could not degrade short-chain degradation products. Therefore, nonylphenol diethoxylate and its carboxylated derivative [nonylphenoxy(ethoxy)]acetic acid were accumulated during the test [73]. Similarly Xin et al. used activated sludge for the isolation of bacteria capable of nonylphenol ethoxylate degradation [74]. The authors used improved the cultivation method according to Tamaki et al. [76] using gellan gum instead of agar, which enables obtaining diverse isolates. Using this method Xin et al. isolated eight bacterial strains belonging to seven different genera, i.e., *Pseudomonas* NP15b, *Sphingomonas* NP41b, *Sphingomonas* NP42a, *Sphingobium* NP22b, *Cupriavidus* NP213b, *Ralstonia* NP47a, *Achromobacter* NP31a, and *Staphylococcus* NP426a [74]. These strains displayed four biodegradation patterns A, B, C, and D. In pattern A *Sphingobium* NP22b, *Sphingomonas* NP41b, *Cupriavidus* NP213b, and *Staphylococcus* NP426a strains degraded nonylphenol ethoxylates to short-chain ethoxylates (mainly nonylphenol di- and triethoxylates) and their carboxylated derivatives. In pattern B *Achromobacter* NP31a and *Pseudomonas* NP15b were able to degrade nonylphenolethoxylates down to nonylphenol monoethoxylate and its carboxylaled derivative. On the other hand, *Sphingomonas* NP42a in pattern C produced short-chain nonylphenol ethoxylates with nonylphenol monoethoxylate being the main product, while only small amount of carboxylated derivatives was found. Finally, strain *Ralstonia* NP47a (pattern D) was capable only of oxidizing nonylphenol ethoxylates without any chain shortening [74].

Biodegradation of octylphenol ethoxylates by isolated bacterial strain was tested by Hotta et al. [75]. The authors isolated *Sphingomonas* sp. strain BSN22 from bean fields. The isolate was capable

of biodegradation down to octylphenol. The mechanism involved chain shortening down to octylphenol triethoxylate, which was then transformed to [octylphenoxy(ethoxy)]acetic acid and finally to octylphenol which was accumulated. What is more important, the authors not only defined the biodegradation route but also showed significant importance of metal ions in the process. It was presented that the presence of Ca^{2+} and Mg^{2+} ions was essential for the biodegradation and addition of Fe^{3+} ions significantly improved the biodegradation rate. Nevertheless, this test also showed that further studies have to be performed for successful removal of alkylphenols [75].

These studies were undertaken by numerous scientific groups. Staples et al. tested biodegradation of alkylphenol ethoxylates and alkylphenols using activated sludge from a sewage treatment plant [77]. Successful removal of these contaminants was noted with only traces of alkylphenols left after 35 days in the tests with alkylphenol ethoxylates and no alkylphenols in the tests with pure octylphenol and nonylphenol. However, bacteria responsible for this successful process were not isolated [77].

Soares et al. tested biodegradation on nonylphenol trying to isolate bacteria from soil, a municipal wastewater treatment plant and a former manufactured gas plant [78]. By contrast to the results obtained by Staples et al. [77] there was no biodegradation of nonylphenol by bacteria from the wastewater treatment plant. There was no strain isolated from the manufactured gas plant, either. However, the authors could isolate three different strains of bacteria from the nonylphenol contaminated soil. Two strains were identified as *Pseudomonas* spp. and one as *Stenotrophomonas* sp. All these strains were capable of degrading nonylphenol in single as well as in mixed colonies with *Stenotrophomonas* sp. strain being the most efficient [78].

Tanghe et al. performed purification of nonylphenol degrading cultures from activated sludge and isolated two *Pseudomonas putida* strains [79]. Their repeated incubation resulted in both positive and negative results of nonylhenol degradation. Only prolonged incubation resulted in isolation of a slow growing *Alcaligenes* sp. strain. As was proved in further tests, only this strain was able to degrade nonylphenol and the two *Pseudomonas* spp. strains previously isolated could degrade nonylphenol only in mixed colonies with *Alcaligenes* sp. strain. On the other hand, all three strains were capable of degrading octylphenol and none was able to degrade long-chain alkylphenol ethoxylates. However, as biodegradation of the ethoxy chain is not problematic for many bacteria, studies on alkylphenols are still the most important to remove endocrine disrupting chemicals formed during primary biodegradation of alkylphenol ethoxylates [79].

Detailed analysis of bacterial degradation pathways and genes involved in biodegradation of alkylphenols was presented by Tuan

et al. [80]. The authors isolated 18 different bacterial strains capable of degrading octylphenol. These bacteria belonged to six genera, i.e., 12 strains of *Pseudomonas*, two strains of *Alcaligenes*, and one strain of each *Acinetobacter*, *Inquilinus*, *Methylobacterium* and *Shinella*. The biodegradation pathways described in the study involved initial multicomponent phenol hydroxygenase with the formation of alkylcatechol and further oxidative ring cleavage by catechol 1,2-dioxygenase or catechol 2,3-dioxygenase leading to alkyl *cis,cis*-muconate and alkyl 2-hydroxymuconic semialdehyde, respectively. Genes responsible for these degradation steps were identified using polymerase chain reaction (PCR) and sequencing. The results also showed that horizontal gene transfer for genes encoding the key enzymes involved in identified biodegradation pathways might play a major role in adaptation of microbial communities to environmental contamination by alkylphenols [80].

Problems with searching for bacteria degrading alkylphenols led to a growing number of studies on their fungal biodegradation. Fungi were found to degrade alkylphenols quite easily and a number of studies reported successful degradation of alkylphenols [81–83]. Fungi used in these studies were isolated from different natural sources and generally can be divided into water fungi and white rot fungi. Junghanns et al. tested aquatic fungi isolated from nonylphenol polluted surface water [81]. Degradation efficiency of UHH 1-6-188-4 strain isolated from a river was higher than that of *Clavariopsis aquatic* strain isolated from a brook. Almost complete primary biodegradation was achieved after a month using UHH 1-6-188-4 strain while only about 60% of nonylphenol was degraded by *Clavariopsis aquatic* strain at similar time. Among biodegradation products two different types of compounds were found—more abundant products with hydroxyl group in alkyl chain and less abundant compounds with shortened alkyl chain. Laccases from the two fungi were also isolated and used in degradation tests of branched and linear nonylphenol. Usage of laccase from UHH 1-6-188-4 strain led to better results than those found for laccase from *Clavariopsis aquatic* strain, e.g., degradation of branched nonylphenol was 63.5% and 14.0% for the two respective laccases after only 24 h. However, it must be underlined that addition of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) to each laccase as the artificial model redox mediator resulted in almost complete primary degradation of nonylphenol in 24 h. The degradation products formed were of higher molecular masses which indicates to oxidative coupling taking place in the process [81]. Similar formation of polymeric products was found by Tsutsumi et al. [84] who tested degradation of nonylphenol and bisphenol A by fungal laccase with and without 1-hydroxybenzotriazole playing role of the so-called laccase-mediator system as well as by manganese peroxidase from white rot fungi. What is more important, Tsutsumi et al. proved

considerably lower endocrine disrupting activity of the degradation mixtures after only a few hours [84].

Comparatively fast degradation was obtained by Moon and Song who isolated fungi from various forest soils [82]. Most of nine isolated strains could degrade alkylphenols to high extent but only one degraded these compounds during 1 day. The white rot fungus *Irpex lacteus* strain was subjected to further testing and its manganese peroxidase was successfully isolated. Usage of purified enzyme led to over 99% primary degradation of octylphenol in 90 min and over 90% primary degradation of nonylphenol in 60 min. Estrogenic activity was also reduced but only to about 10–20% of the initial values. This inconsistency was attributed to residual recalcitrant degradation products that were identified as 9-hexadecenoic acid, 9-octadeceamide, hexadecanoic acid, tetradecanoic acid, methyl-cis-9-octadecenoate, and 3,4-dimethoxybenzoic acid methyl ester [82].

Subramanian and Yadav tested another white rot fungus strain—*Phanerochaete chrysosporium* [83]. The strain degraded nonylphenol in both nutrient-limited and nutrient-rich cultures. Essential role of P450 monooxygenases was found under nutrient-rich conditions, while under nutrient-limited conditions with low nitrogen content enzymes involved in degradation could not be precisely defined. The study showed that P450 genes in *Phanerochaete chrysosporium* are induced by nonylphenol in a nutrient-specific manner. Moreover, there were only two genes (induced solely in nutrient-rich culture) that showed extraordinarily high levels of induction and might be involved primarily in degradation of nonylphenol. Therefore, although the P450 genes are present in many organisms, rarity of genes strongly induced by nonylphenol clearly shows problems with appropriate enzymes functioning as a terminal oxidase. This is obviously connected with highly branched nature of the most widely used technical nonylphenol [83].

2.3 Biodegradation of Phthalates

Phthalates belong to probably the most widely used EDCs due to their widespread application as plasticizers in plastics. As these compounds are not covalently bonded to polymers, they can be easily leached to water. Primary biodegradation of phthalate diesters leads to phthalate monoesters and then to phthalic acid. Both phthalic acid and the leaving groups can be further oxidized and enter tricarboxylic acid (TCA) cycle (Fig. 5) [85]. However, even the first steps in this biodegradation are problematic. As it was presented by Barnabé et al. biodegradation of phthalates in the sewage treatment plant is not complete—80% of DEHP found in the sewage influent was assayed in the effluent [86]. Furthermore, 15% of DEHP was found in dewatered sludge. Even though the samples were collected on the same day and error in the estimation of the biodegradation can be high (due to retention in the sewage treatment plant), still estimation of DEHP removal shows huge

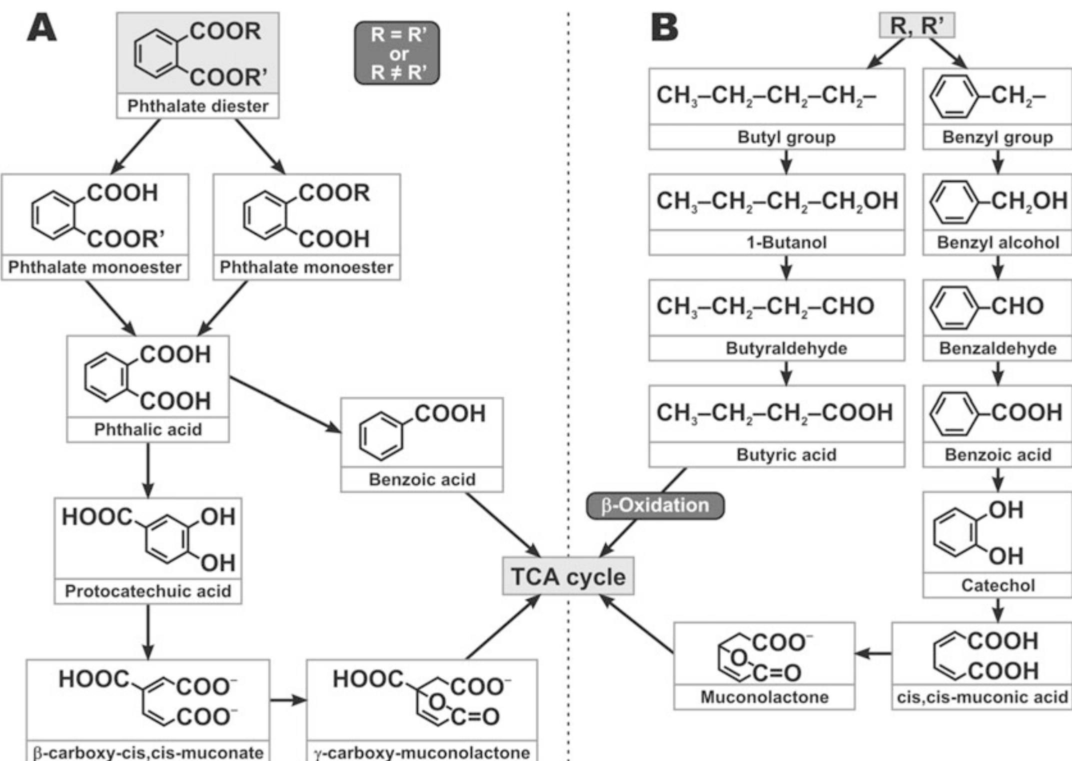

Fig. 5 A biodegradation scheme of phthalates. (**a**) degradation of the main structure. (**b**) degradation of exemplary substituents

problems with its degradation. Moreover, while concentration in the effluent was 54 μg/L, it was much higher in the receiving river, i.e., 180 μg/L and in its sludge—110,000 μg/kg [86]. High contamination of the river water shows widespread contamination and much higher contamination of the sludge focuses our attention on accumulation of phthalates.

Due to problems with high accumulation of phthalates in sludge and soil studies were undertaken to remove these contaminants [87–89]. Chang et al. tested removal of DBP and DEHP from compost-amended soil [87]. The authors found that biodegradation of DBP and DEHP in soil by indigenous microorganisms led to the removal of these contaminants in 9 days and 15 days, respectively. Addition of compost substantially accelerated biodegradation to 5 days and 9 days for DBP and DEHP, respectively. Yuan et al. tested removal of the same two phthalates from sewage sludge-amended soil [88]. Removal of DBP and DEHP from sludge was dependent on both their concentration and size of sludge particle. Tests at higher concentrations and with bigger particles resulted in slower biodegradation of both these contaminants. Proportion of soil to sludge was also important with 1:0.2 soil to sludge ratio resulting in the fastest biodegradation of both

the compounds. A change in the soil to sludge proportion resulted in reduction of the biodegradation half-life from 1.6 to 1.0 day and from 8.7 to 5.8 days. The authors also isolated five bacterial strains responsible for fast degradation of bisphenols (*Rhodococcus* sp., *Bacillus horti*, *Microbacterium* sp., *Mycobacterium* sp., *Streptomyces* sp., and *Rhodococcus* sp.) for which biodegradation half-life was from 0.3 to 1.4 days and from 1.6 to 4.2 day for DBP and DEHP, respectively, with the best degrading strain being *Rhodococcus* sp. [88]. It is worth mentioning that *Rhodococcus* sp. was previously reported to show excellent degradation activity toward dimethyl phthalate (DMP), diethyl phthalate (DEP), and DBP [90]. However, usage of sewage sludge for bioremediation of phthalates-contaminated soil is not always successful. Ferreira et al. tested removal of five different phthalates and only 60–85% of phthalates were removed during 120 days [89].

Similarly to studies on bisphenols and alkylphenols experiments on phthalates include isolation of bacteria form different sources. Although selection of bacterial strains started in the 1970s, still many publications on this topic can be found nowadays but the newly published studies frequently include more precise information like identification of genome fragments responsible for biodegradation and characterizations of enzymes responsible for this process. Moreover, some studies present optimization of biodegradation conditions with the use of statistical methods. Bacteria able to degrade phthalates are isolated from different sources, mainly from soil [91–94] but also from natural waters [95], wastewater [96], and activated sludge [90, 97].

Chao et al. used soil from a river bank and obtained twelve isolates degrading DBP [91]. Eight isolates degraded more than 90% of DBP in 3 days out of which five were qualified as fast degrading and three as medium degrading. Bacterial strains were identified only in 8 out of 12 isolates. Then PCR amplifications and nucleotide sequencing were used for the identification of DNA *phtA* fragment, i.e., the subunit of phthalate dioxygenase gene. The *phtA* fragment was found only in six strains, each belonging to fast and medium DBP-degrading bacteria but surprisingly it was not identified in two fast-degrading strains. It was supposed that a different kind of oxygenase had to be present in the two strains [91].

Chen et al. isolated one DBP-degrading strain from soil taken from a treated sewage disposal area [92]. The strain was named M11 and it was most closely related to *Camelimonas lactis* strain M2040. It could degrade phthalates only partially, i.e., leaving 44% of DBP, 28% of diethyl phthalate (DEP), 13% of diphenyl phthalate, and 72% of di-*n*-amyl phthalate after 72 h. No degradation of dimethyl phthalate (DMP) took place but still isolated strain was able to degrade medium-chain phthalates. Moreover, the authors managed to amplify the DBP hydrolase gene by PCR, ligated to appropriate expression vector, and transformed to *Escherichia coli*

cells. This enabled characterization of DBP hydrolase activity including its activity at different pH and temperature [92].

Prasad and Suresh isolated *Variovorax* sp. strain from garbage dumped soil [93]. The strain was able to utilize mixture of DMP, DEP, and DBP as well as the individual phthalate esters as the sole source of carbon and energy. Removal of these phthalates was accomplished in <30 days. The authors also isolated the crude cell free extract from their mid exponential growth phase. Degradation of DMP and DEP using the extract was considerably accelerated—about 80% degradation was noted during an hour [93].

Surhio et al. also used soil as a source of phthalate-degrading bacteria [94]. Although no identification of genome or enzymes responsible for biodegradation was done, the publication is worth mentioning because the isolated *Bacillus thuringiensis* strain was able to fully degrade DMP in 72 h at concentration up to 400 mg/L. Furthermore, it could also degrade about 80% of DMP at a concentration 1000 mg/L [94].

Jin et al. isolated *Sphingobium* sp. bacteria from estuary water which enabled biodegradation of a series of phthalic acid esters including DMP, DEP, DBP, di-*n*-octyl phthalate (DOP) [95]. The strain could also utilize monobutyl phthalate but could not use phthalic acid. Therefore, even though the strain could rapidly degrade phthalates and tolerate NaCl up to 4%, it was clearly shown that it needs another bacterial strain to complete biodegradation of phthalates. In another paper, Jin et al. presented results of studies on *Achromobacter* sp. bacteria isolated from wastewater obtained from a rural sewage treatment plant [96]. The strain could utilize DMP, DEP, DBP, DEHP, and DOP. It was also shown that the strain preferred growing on long alkyl chain phthalates. The study identified presence of one plasmid in the cell. This plasmid controlled biodegradation of phthalates and its removal led to loss of degrading capabilities of isolated *Achromobacter* sp. strain.

Lu et al. isolated *Rhodococcus* sp. bacterial strain from activated sludge [90]. The strain was able to degrade DMP, DEP, and DBP. The authors used statistical design methods for optimization of biodegradation conditions with a four-level three-factorial design. Under optimized conditions the strain could degrade phthalates during 6 days at concentration up to 100 mg/L [90]. Li et al. also isolated bacterial strain from activated sludge [97]. The strain was identified as *Serratia marcascens* and was able to degrade DBP. The authors optimized the biodegradation conditions using the Taguchi method with a three-level three-factorial design. Under optimized conditions the strain could degrade DBP during 6 days at concentration up to 100 mg/L [97].

Biodegradation of phthalates was also studied with the use of fungi [98–101]. Chai et al. tested biodegradation of DEHP using 14 different fungi isolated from different soils or rice grains [98]. Nine of these strains degraded DEHP in more than 50% forming *o*-

phthalic acid, 2-ethylhexanol, and 2-ethylhexanoic acid. The most effective in degradation were *Fusarium* species including *Fusarium graminearum* NFRI-1280, *Fusarium morniforme* 2-2, and *Fusarium sporotrichioides* NFRI-1012 [98].

Soon-Seok et al. investigated ten white rot fungi species and tested biodegradation of DMP, DEP, and BBP [99]. Depending on media up to seven species could degrade the three phthalates in 12 days or less with *Pleurotus ostreatus* degrading phthalates in 2–8 days. The authors also noted considerable and fast decrease in estrogenic activity of DMP during biodegradation showing great potential of fungi [99].

Luo et al. tested biodegradation of different phthalate isomers by *Fusarium* sp. DNT-5-3 fungi which selectively degraded these compounds [100]. Two methyl groups could be removed from the *para* isomer, one from the *meta* isomer and there was no degradation of the *ortho* isomer. Further studies performed by the same scientific group were devoted to isolation of intracellular esterases catalyzing hydrolysis of two phthalates [102, 103]. The proteins were isolated and then purified by ion exchange chromatography. It can be easily compared that the two isolated esterases [102, 103] have different molecular masses. Moreover, there was a difference among degradation capabilities of the two isolated proteins [102, 103] and *Fusarium* sp. DNT-5-3 fungi (without isolated enzyme) [100]. It was found that the esterases involved in biodegradation of phthalates are highly substrate-specific. The esterase isolated with the *para* isomer as a substrate could hydrolyze only dimethyl *para*-phthalate (without other isomers and monomethyl phthalates) [102] and the one isolated with the *meta* isomer could hydrolyze both dimethyl *para*-phthalate and dimethyl *meta*-phthalate (but still no monomethyl phthalates) [103]. The results from the three studies clearly showed that there must be another esterase able to degrade monomethyl *para*-phthalate degraded by *Fusarium* sp. DNT-5-3 fungi [100, 102, 103].

Studies on biodegradation of phthalates included also genetic modification of fungi. Kum et al. transformed a laccase expression vector from *Phlebia tremellosa* to *Irpex lacteus* fungus to enhance its biodegradation capabilities [101]. To achieve this aim a laccase gene from *Phlebia tremellosa* was inserted at the *Bam*H I site of pBARGEM7-1 plasmid to construct a laccase expression vector (pBARPprolac) which was then inserted into *Irpex lacteus.* Stable integration was confirmed using PCR and then two selected transformants were tested for their laccase activity. They were grown on several endocrine disrupting compounds including BBP, DEP, as well as bisphenol A and NP. Laccase activity of the transformants was induced a few times more than that from the unmodified *Irpex lacteus.* The use of the transformant in degradation of BBP and DEP led to more than 90% removal of these compounds on day 3 of the experiment. Increased removal rate of

estrogenic activity compared to the unmodified *Irpex lacteus* was also observed [101].

3 Future Prospects

This review on the biodegradation of bisphenols, alkylphenols, and phthalates clearly shows two trends. On the one hand, latest studies aim at isolation and full characterization of bacteria. Genes responsible for degradation of specific compounds or production of specific enzymes are often identified. These genes can then be transferred to other strains to improve their degradation capabilities. This approach can substitute usage of mixed bacterial cultures where different often pathogenic species are responsible for consecutive steps leading to complete degradation.

On the other hand, similar studies are carried out on fungi. These species degrade EDCs using different and often faster schemes but it must be taken into account that most fungi are difficult to remove once they were used. What is more, some fungal species produce mycotoxins and their usage is not safe. Therefore, studies were undertaken to isolate enzymes responsible for biodegradation of EDCs. They are not only safer but also degrade much faster than fungi. So far relatively low stability of enzymes and their high cost have not enabled their widespread usage. Nevertheless, the latest studies on immobilization of enzymes on polymeric beads due to very high stability of bound enzymes open a new era in biodegradation of EDCs.

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Chapter 2

Biodegradation and Inhibitory Effects of Antibiotics on Biological Wastewater Treatment Systems

Zeynep Cetecioglu and Merve Atasoy

Abstract

Antibiotics are one of the most consumed drugs and have become new emerging pollutants in the environment as antibiotics lead to long-term adverse effects on the ecosystem. They are produced by bacteria to inhibit the growth of other bacteria in nature as a defense mechanism. Furthermore, after discovering their therapeutical features, synthetic production methods were developed. In general, antibiotics are widely used in human medicine, veterinary medicine, farming and aquaculture for the prevention and treatment of diseases. Approximately 90% of the consumed antibiotics are excreted via urinary or fecal pathways from the human body after partial, or no metabolism, and they are transferred to the domestic sewage plants or directly to the environment. Conventional biological treatment of domestic sewage provides very low—if any—reduction for the antibiotics, which usually by-pass treatment and accumulate in the receiving waters, sediments, plants, and animals. The concentration of these materials in domestic wastewaters and surface waters is observed in a range between 0.3 μg/L and 150 μg/L. However, pharmaceutical plants, hospitals, concentrated animal feeding operations, and aquaculture generate effluents having much higher antibiotics concentrations in the range 100–500 mg/L. Consequently, it is essential to gather information on the fate and effect of these compounds at high concentrations for setting the basis for related practical treatment schemes.

Inhibitory action of the antibiotics is experimentally evaluated in two different approaches: Short-term (acute) and long-term (chronic) tests: Acute experiments involve a microbial community selected and sustained by the selected organic substrate in the system and not previously exposed to the inhibitor. In long-term experiments with continuous feeding of the inhibitor, the test may reflect, aside from changes in substrate removal and utilization, adaptation and/or resistance of the microbial community or even shifts in microbial composition in response to continuous exposure to the selected inhibitor. However, a full insight on the inhibitory action can only be acquired when the response of the microbial community is tested for both acute and chronic inhibition impacts.

In this chapter, the most commonly used antibiotic classes such as β-lactams, tetracycline, macrolides, sulfonamides, quinolones are examined. Their fate and transformation during wastewater treatment as well as their inhibitory and toxic effects on the microbial community are discussed by using various toxicity and inhibition tests.

Key words Antibiotics, Biodegradation, Inhibition, Toxicity, Biodegradability, Wastewater treatment

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1 Introduction

"Antibiotic Era" has been started due to the remarkable reduction of the mortality rates of socially and epidemiologically infectious diseases. Antibiotics have been used for treating severe infections, preventing infections in surgical patients, protecting cancer patients and people with decrepit immune systems since the 1940s [1]. In addition, antibiotics are used to prevent and treat diseases in animals as well as to promote the growth of antibiotics that can be classified according to different criteria such as chemical structure, action mechanism, or spectrum. Most important antibiotics can be categorized such as β-lactams (penicillin, cephalosporin, carbapenem), quinolones, tetracyclines, macrolides, sulfonamides, aminoglycosides, and glycopeptides according to their chemical structure [2, 3]. Additionally, antibiotics can be classified according to their target or mechanisms of action into five main groups: (1) to inhibit bacterial cell biosynthesis, (2) to selectively block 30S or 50S ribosomes, (3) to block the DNA replication, (4) to disrupt the integrity of cell membranes, and (5) to antagonize metabolic processes.

According to the antibiotic consumption statistics, global antibiotic consumption rate increased more than 30%, between 2000 and 2010 years [4]. Moreover, approximately 50% of antibiotic consumption rate is the result of improper usage. Antibiotic consumption rate depends on various factors such as easy access to antibiotics, expanding insurance coverage, seasonality, economic growth, usage for not only medical aims but also to increase growth promotion of animals [4].

Antibiotics, massively used for both human and veterinary medicine, are discharged into the environment via urine and feces. Urine and feces are rarely used directly as fertilizer, but they are mostly received to wastewater treatment plants (WWTPs). Thereby, WWTPs are a primary hotspot for antibiotics before they are released into the environment [5, 6].

Antibiotics can be degraded by microorganisms and/or photocatalytic reactions or eliminated by sorption onto the sludge at WWTP. During biodegradation process, antibiotics can be degraded into by-products that called transformation products (TPs) which are more polar than the main compound. Due to high polarity resulted in low biodegradability, some transformation compound cannot eliminate efficiently. Some of TPs can be more stable and toxic than main compounds [7]. Since most conventional WWTPs are not designed to eliminate highly polar contaminants, antibiotics and transformation products can reach the environment also after treatment. Thereby, they can reach receiving water bodies such as surface waters, ground waters, potentially drinking waters, and also soil. Therefore, these compounds have

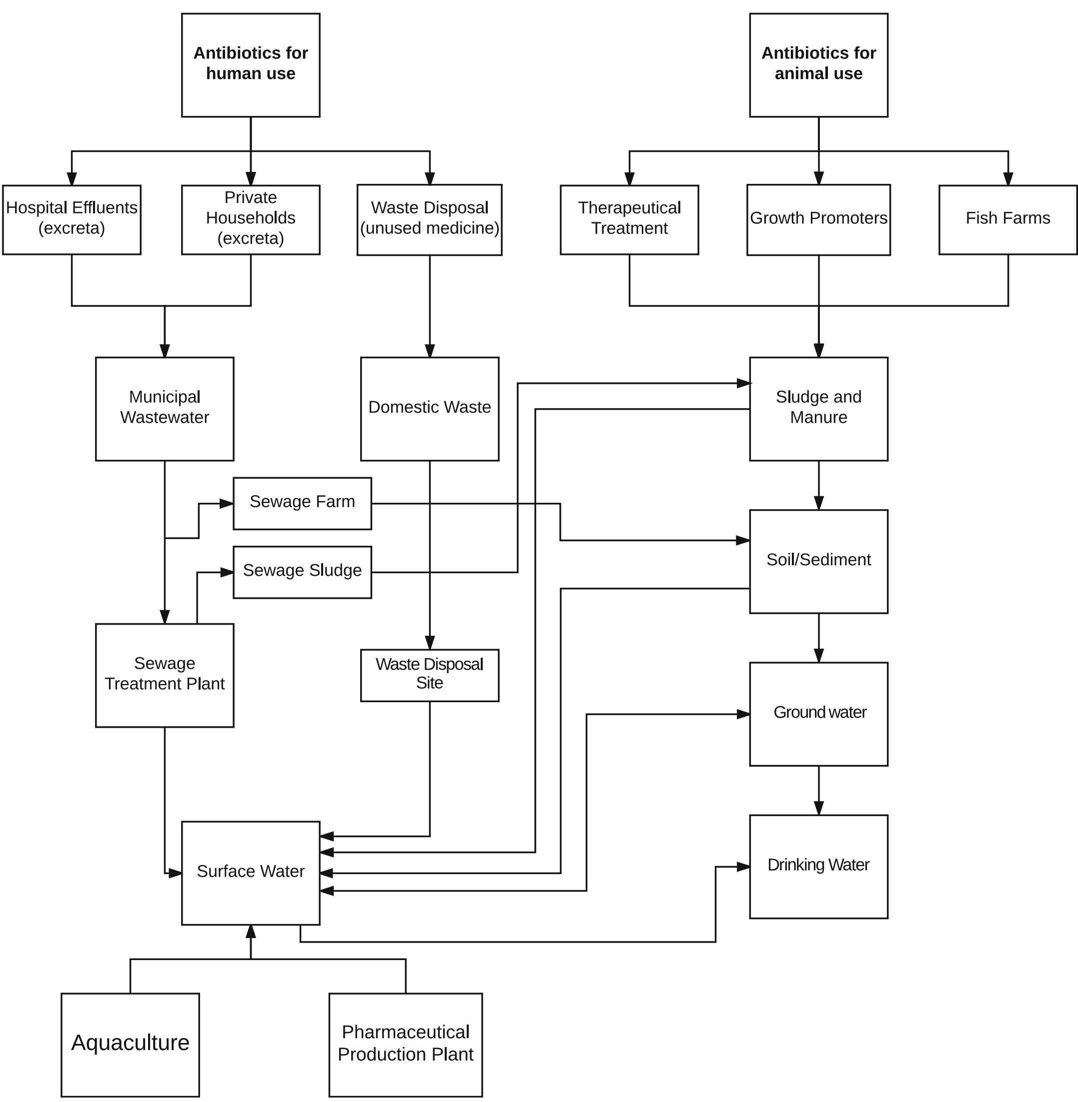

Fig. 1 Fate and transport of antibiotics in the environment (Adapted from Heberer, 2002) [8]

various exposure pathways to the environment after treatment, which is given in Fig. 1.

The result of a direct discharge of wastewater containing untreated antibiotics to surface water or groundwater, landfill leachate, leaking sewers disposal in agricultural areas and other discharges cause antibiotics to entry and accumulate into the environment. Thus, the spread of antibiotic may cause antibiotic resistant bacteria and antibiotic resistance genes (ARGs) into the ecosystem [9]. Although antibiotic resistance genes can occur naturally in the environment, mostly they arise through genetic mutation or by species acquiring resistance from others [10, 11].

According to the U.S. Centers for Disease Control and Prevention (CDC), antibiotic resistance is responsible for more than 2 million infections and 23,000 deaths each year in the U.S. that estimates economically 35 billion dollar losses in 2013 [12]. In addition, antibiotic-resistant infections caused approximately 25,000 deaths and 1.5-billion-euro economic loss at 2009 in Europe [13]. Consequently, about 700,000 people die as a result of antibiotic resistance infections annually at a whole of the world [4].

Besides the adverse effects of antibiotics on nature such as the distribution of antibiotic resistance bacteria and genes, they cause problems in treatment plants as well. The adverse effects of antibiotics on wastewater treatment plant depend on the type of processes and operation conditions. In general, a conventional WWTP consists of filtration, coagulation/flocculation, and precipitation as a physio-chemical process, biological process, and sometimes advanced treatment stage. Biological treatment, which is the process where antibiotics are most eliminated, in general consists of activated sludge that enables organic matter biodegradation by microorganisms [2, 8].

Antibiotics have three main routes during biological degradation in WWTPs; they can (1) mineralize, (2) transform to more hydrophobic compounds, (3) transform to more hydrophilic compounds that discharge into the surface water without treatment.

Antibiotics cannot be effectively eliminated by biodegradation alone, even though the low concentration of the toxic compound and many different microorganism species in active sludge contains. One of the possible reasons for poor treatment of antibiotics in WWTP is that very low concentrations of antibiotics may be inadequate for enzymes that are required for degradation of antibiotics. Another one is related to toxic effects of antibiotics. They can inhibit the growth or metabolism of microorganisms. Likewise, biodegradation of antibiotics relies on the structure of compound and properties of WWTPs such as operation conditions [14]. As a result of these causes, antibiotics can adversely affect the nitrogen transformation, methanogenesis, and sulfate reduction during biological treatment at WWTPs [15].

All the aspects of the adverse effects of antibiotics, which are damaging to either the survival or normal function of microorganisms or their communities, on microorganisms are determined by toxicity tests. According to the endpoint of measurement, toxicity tests are classified as acute and chronic [2, 16].

In acute toxicity tests, toxic effects of exposure to a chemical on microorganism community, which are not previously exposed to the chemical, are determined. A typical acute toxicity test exposes test organisms to a series of dilutions of the compound and records deaths over a period, typically 24–96 h.

The results can be analyzed by comparing the percentage of deaths of organisms exposed to the chemical to the percentage of

organisms exposed to uncontaminated media. The results are reported in terms of effective concentration, EC50 or EC20, which is the concentration of 20% and 50% reduction in measured endpoints, but the respiration rate, enzyme activity, substrate utilization rate, oxygen consumption, microbial growth rate, bacterial luminescence, and biogas production rate are used as an indicator to measure inhibition effects [16–18].

The chronic toxicity tests are also expressed as long-term tests that measure the effects of exposure to lower, less toxic concentrations. The test is considered chronic if it contains more than 10% of the organism's lifespan; otherwise, it is like an acute toxicity test. The most remarkable distinctness between chronic and acute tests is that chronic tests include acclimation period of the microorganism. Chronic tests are long-term tests that take days, weeks, or months, including at least two generations of test organisms. As a result of the application of continuous low concentrations, the end point of the test is determined according to the sub-lethal effects (growth retardation, reproductive failure, nerve dysfunction, lack of motility, developmental tastes with behavioral changes and structural abnormalities) or both death and lethal effects. In addition, NOEC (observed effect concentrations) or LOEC (lowest observed effects concentration) can be thought of as end points of the test [19–21].

The toxic or inhibitory effects of compounds on the microbial community as well as on the environment can be examined by various types of tests. Most of these tests are standardized by The Organization for Economic Co-operation and Development (OECD), International Organization for Standardization (ISO), and Environmental Protection Agency (EPA), which are defined in detail how a test should be designed, executed, and reported. Even though standardized tests are used generally, there are also non-standard test methods, in contrast, any other test method, designed case-by-case depending on the research question, and typically developed and performed by academic research groups [17, 21].

OECD recommends validated test methods for characterizing the fate and behavior as well as the effect of chemical substances for regulatory purposes. Mostly used OECD tests for the effects of chemical substances on aquatic organisms OECD (2014) are OECD TG 209 (2010) activated sludge, respiration inhibition test; OECD TG 224 (2007) detection of the inhibitory effects on anaerobic bacteria activity that is gas production reduction of anaerobically digesting (sewage) sludge [21].

The ISO have been developed primarily for testing the effects of environmental water samples. Mostly, ISO 11348 determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test), ISO 10712 pseudomonas putida growth inhibition test (pseudomonas cell multiplication

inhibition test) are used for the determination of the effects of water and wastewater on bacteria [21].

This chapter aimed to discuss the behavior of antibiotics in the biological wastewater treatment plants in terms of fate and effects of the main compound, transformation products, and metabolites on the microbial community by using various toxicity and inhibition test.

2 Fate of Antibiotics in the Environment

There are three main potential routes based on the fate of antibiotics in the environment; they can completely be eliminated by microorganisms (bacteria, fungi, algae, etc.) or they can be degraded partially, or they can persist in the environment. The elimination process in nature can be biotic that is biodegradation or abiotic which includes adsorption, hydrolysis, photolysis, thermolysis, oxidation/reduction reactions.

The fate of antibiotics in the environment depends on the physicochemical properties of the compound and ongoing processes, as well as on environmental factors (climate, pH, redox condition, water, and sediment components) [22].

2.1 Biodegradation

Antibiotics can be biodegraded by microorganisms in surface water, groundwater, soil and sediments, sewage system. However, most of the antibiotics are persistent to biodegradation, but some of them used for human medicine are easily degradable [23].

2.2 Adsorption

Some antibiotics such as tetracyclines are eliminated by sorption into soils and sediments, instead of biodegradation or photodegradation, in nature. The efficiency of the sorption process depends on the pH, organic matter concentration, minerals of soil and distribution coefficient of a compound [23, 24].

2.3 Photodegradation, Hydrolysis, and Thermolysis

Antibiotics can be also degraded by either photodegradation or hydrolysis or thermolysis processes instead of biodegradation. In photodegradation, light-sensitive antibiotics decompose as a consequence of light exposure. Likewise, biodegradation and photodegradation, another elimination process, is hydrolysis that allows the decomposition of antibiotics due to water instability [23, 25].

2.4 Oxidation/Reduction Reactions

Advanced oxidation/reduction processes (AO/RPs) are basically defined by the production of ●OH as an oxidant and either hydrated electron (e^- aq) or a hydrogen atom (H●) as reductants. Relatively few studies have conducted about the degradation of antibiotics by AOP oxidation, with the exception of photo-Fenton, or UV/TiO_2 processes [26].

3 Removal of Antibiotics in the WWTPs

The major removal pathways of antibiotics at wastewater treatment plant include adsorption, biodegradation, and oxidation processes. In addition, other removal pathways, such as hydrolysis, photolysis, and volatilization, may be neglected due to their insignificant role for antibiotics reduction in wastewater treatment processes. The conventional WWTP comprise primary treatment that used to eliminate solid contents, the secondary treatment that commonly relies on the biological processes for degradation of organic matters, and tertiary also called advanced treatment includes nitrogen, phosphorus, and/or other pollutants removal processes and disinfection by chlorination, ozonization, or ultraviolet irritation. The advanced treatment step does not consist of WWTPs as usually [27–29].

3.1 Biological Treatment Process

The mechanism of the biological treatment depends on microbial activity; they use organic compound and nutrients to gain energy and as building blocks for their growth.

Despite activated sludge including high density and wide range consortium of microorganisms, antibiotics cannot be efficiently eliminated in biological treatment processes. The reason for the poor treatment of antibiotics depends on the three major points: (1) the relatively low concentration of antibiotics in the wastewater leads to lack of enzymes that are responsible for biodegradation of antibiotics, (2) inhibitory or toxic properties of antibiotics that can stop the microorganism activity that is responsible for biodegradation, (3) properties of antibiotics, (4) operation conditions of WWTP.

Many antibiotics have been considered resistant to biodegradation in the literature, but recent studies have shown that antibiotics may be biodegradable under different operational conditions, such as respirometric conditions (aerobic, anaerobic, anoxic, or their combination), solid retention time (SRT), hydraulic retention time (HRT) [19, 30, 31].

Moreover, while some antibiotics may be partially degraded, most antibiotics such as ceftriaxone, ciprofloxacin, ofloxacin, metronidazole, and trimethoprim are not biodegradable. Even if antibiotics can be biodegraded, its transformation compounds would be more toxic and persistent than the parent compound, such as sulfamethoxazole [2].

Additionally, many antibiotics and their transformation products have adverse effects on microbial community in the biological systems such as aerobic/anaerobic degradation, nitrification, sulfate reduction, phosphate removal, etc. [15]. For example, in the nitrification process, antibiotics such as ofloxacin and sulfamethoxazole inhibit the microorganisms that are responsible for converting of nitrite to nitrate. Thus, nitrite nitrogen that is particularly

toxic to the system can be accumulated in the system. Similarly, antibiotics such as benzylpenicillin and metronidazole can inhibit the microbial activity of acetoclastic methanogens at the methanogenesis phase of the anaerobic digestion [32]. Nevertheless, macrolides such as erythromycin have not any effect on biological processes.

In addition to inhibitory effects of antibiotics, they also cause the alteration of the microbial community in the activated sludge [33].

3.2 Adsorption Process

Adsorption is the process to accumulate compounds from liquid or gas phase onto the surface of adsorbent by physical and/or chemical processes that allow removal, conditioning, and remediation of organic and inorganic hazardous materials from wastewater. Antibiotics are eliminated with activated sludge and adsorbents such as activated carbon, biochar, carbon nanotubes (CNT), clay mineral (bentonite), and ions exchange resins in the adsorption process, which is one of the most important and effective antibiotic treatment methods. The efficiency of the adsorption process depends upon the specific surface area (SSA), porosity, surface polarity, pore diameter, physical shape and the functional group of adsorbent and hydrophobicity, shape, charge, and size of the antibiotic. Furthermore, the initial concentration of antibiotics, the composition of the wastewater, the temperature, and the pH are critical parameters for the efficient adsorption process [27, 34].

In general, the adsorption process comprised of four main steps that are [3]

1. The solute transport in bulk adsorbate action by the still liquid film surrounding the adsorbent,
2. The film diffusion that is adsorbate transport along the film,
3. The pore diffusion that is adsorbate diffusion through the porous structure to the active sites,
4. The adsorption interaction between adsorbate and porous structure.

Many studies have been carried out on the removal of antibiotics by the adsorption process [35–37]. Macrolides, fluoroquinolones, trimethoprim, clindamycin, and amoxicillin are removed substantially by adsorption, while erythromycin and roxithromycin are partially eliminated [38]. Although the adsorption process is essential for the removal of ciprofloxacin and tetracycline into the activated sludge, sulfamethoxazole and trimethoprim cannot be removed by this process [34].

3.3 Advanced Oxidation Processes

Advanced oxidation processes (AOPs) have a broad range of methods for water and wastewater treatment. These methods such as ozonation, chlorination, fenton oxidation, and ultraviolet irradiation (UV) are based mainly on the use and generation of powerful

transitory species, which are mainly the hydroxyl radical (·OH). One of the powerful and effective oxidizing agents is hydroxyl radical that leads to oxidation and mineralization of organic matter [39].

Up to now, many studies have been conducted on the removal of antibiotics with AOPs. According to the result of these studies, most of the antibiotic can be removed by AOPs. However, more toxic and stable transformation products can be formed after.

Ozonation, one of the advanced oxidation processes, has been widely used in wastewater treatment recently, although it has traditionally been used in drinking water treatment. Antibiotics can be oxidized either by ozone (O_3) or by hydroxyl radicals, throughout the ozonation process.

Most of the sulfonamides, β-lactams, quinolones, tetracyclines, lincosamides, trimethoprim, and macrolides can be degraded by ozonation. However, the ozonation process can be critically affected by pH that decreases the reaction rate and absorption rate of ozone [39–41].

Fenton oxidation process relies on the metal (mainly iron) catalyzed oxidation-reduction which is provided by oxidation of many antibiotic classes such as quinolones, trimethoprim, tetracyclines, and β-lactams [27].

Chlorination is the most widely used disinfection system in the world for disinfection of drinking water and wastewater discharge. Studies have shown that chlorination removes not only pathogens but also antibiotics such as roxithromycin, erythromycin, sulfamethoxazole, and trimethoprim [32]. Nevertheless, chlorination causes more toxic and stable transformation products during antibiotic elimination [27, 32].

Ultraviolet (UV) disinfection process includes direct or indirect photolytic degradation according to the occurrence of the adsorbent type. Tetracyclines and ciprofloxacin degrade completely by UV, while penicillin is removed in very low efficiency (Alaton and Dogruel, 2004). After all, UV disinfection is the most promising process for antibiotic elimination in wastewater [27].

Traditional wastewater treatment procedures such as coagulation, flocculation, filtration, and biological treatment do not adequately remove antibiotics, so most antibiotics are discharged after wastewater treatment. For this reason, ozonation, chlorination, fenton oxidation, and ultraviolet irritation have been developed for antibiotic removal. However, the removal of antibiotics in wastewater treatment plants is still an important research area since these systems may require high energy and/or chemical and cause the formation of more toxic and stable conversion products during the elimination process. Therefore, since biological treatment processes have low energy and chemical requirements, they are most promising methods for the treatment of antibiotics in wastewater.

4 Inhibition and Toxicity Tests

In general, antibiotics have adverse effects both on microbial community in WWTPs accordingly the treatment efficiency, and the environment. These adverse effects may be inhibitory or toxic effects. The inhibitory effects could be (1) competitive; the inhibitor prevents binding of the substrate, (2) uncompetitive; the inhibitor prevents the conversion of the product by affecting the enzyme substrate complex, (3) non-competitive; the inhibitor affects free enzyme or the enzyme-inhibitor complex. The binding of the inhibitor is reversible; however, if the enzyme has been made inactive by the binding or if the bound inhibitor does not release, then this is a case of toxicity and not inhibition [42]. Ecotoxicology and toxicology tests have been developed to determine and evaluate these inhibitory and toxic effects. In this chapter, inhibitory and toxic effects of antibiotics on the microbial community during biological wastewater treatment processes are evaluated.

Environmental toxicology tests are designed to use appropriate organisms and sensitive effect measurements in a relevant organism during a particular test period. Moreover, these are conducted to establish a proper relationship between the biological effect and the testing compound.

The type of the environmental toxicology tests can differ from simple bio-tests to whole ecosystem assessment test, depending on the aim and targeted end point of the test.

They can be designed on three general approaches by selecting the endpoints starting from molecular level indicators to the lethality of the test organism, and each has advantages and limitations [43].

Microcosm and mesocosm; they can be defined so as to study the effects in an experimental model ecosystem. Microcosms and mesocosms are multispecies toxicity tests modeling the real ecosystem. Mesocosms are used to examine the natural environment in any outdoor experiment while microcosms are used to model and predict the behavior of natural ecosystems by artificial and simplified ecosystems.

In situ *experiments*: these examine the effects in a natural ecosystem. In situ experiments include the observation of indicating organisms existing naturally that is passive biomonitoring or placed by the assessor into the environment that is also active biomonitoring.

Bioassays: this evaluates the effects to study in a controlled laboratory experiment with a limited number of variables.

Bioassays are used to test acute and chronic toxicity, mutagenicity, carcinogenicity, teratogenicity, and repro-toxicity by simple and single species laboratory test methods. Bioassays cannot stimulate reality in detail, but they represent one single organism, they work with one single chemical substance or a known mixture. Even though small amount of sample is used in bioassays, they are

statistically relevant. In addition, they are easy to standardize because they can be reproducible and comparable with multiple numbers of replicates that may be tested at the same time [22].

Microbial bioassays can be used for environmental monitoring by observing transformations, growth or mortality, respiration, inhibition, and luminescence. Mostly, the microbial bioassays are used to determine antibiotic adverse effects on microorganisms. The method of microbial bioassay depends on antibiotic inhibition of the growth strain of bacteria that grow on agar plates. Various commercial microbial inhibition tests are common because they do not require specialized equipment or harmful solvents and are easy to implement at the same time.

However, the use of confirmatory techniques is always necessary to cause of observed effect cannot be conclusively attributed to one chemical substance due to bioassays are often not specific for an individual chemical substance. For this reason, the use of bioassays for chemical monitoring is becoming increasingly doubtful. Therefore, aquatic toxicology tests are used in recent studies.

Bioassays are used to determine the effect of a toxic compound on an organism, whereas toxicity tests are used to find out the concentration of the toxic compound. Nevertheless, these two terms bioassay and toxicity tests are considered synonymously.

4.1 Toxicity Test

Historically, toxicity tests are conducted according to the following systems: in vitro and in vivo screening level tests, short-term tests with single species, long-term tests with single species, and then tests with multispecies [17].

Most single species tests are conducted in the laboratory. These tests can provide considerable information on the external and internal concentrations of chemicals and exposure time that produce changes in mortality, growth, reproduction, pathology, behavior, physiology, and biochemistry of organisms within species. Nevertheless, the results are rarely used to evaluate the chemical effects on the biological organization [21].

Single-species tests easily provide the cause-and-effect relationships because of the degree of control over laboratory conditions. Also, these tests are straightforward and relatively simple to conduct. Moreover, these tests are carried out with individual species as considered representative of broad classes of organisms, so that the results provide toxicity effect of specific chemicals on different types of organisms under given conditions. However, the effects observed in the laboratory may not occur in the same way or to the same degree at similar concentrations in the natural environment [43].

4.2 Toxicity Effects

Adverse effects on microorganisms are induction or inhibition of enzymes and/or enzyme systems and their associated functions.

In determining the toxic and inhibitory effects of a new chemical on microorganisms, an acute toxicity test is the first conducted to assess the average lethal concentration (LC50) of the compound in the water to test organisms that are exposed. The LC50 is the concentration estimated to produce mortality in 50% of a test population over a particular period. The length of exposure is usually 24–96 h, depending on the species. If effects other than mortality are measured, the expression average effective concentration (EC50) is used, which is the concentration of a chemical estimated to produce a specific effect such as behavioral or physiological in 50% of a population of test species after a specified length of exposure time (24 or 48 h) [21].

4.3 Description of the Tests Methods

The aquatic toxicology test can be categorized according to exposure time, test situation, and type of microorganisms to be tested. The test may give information to determine no observed effect concentration (NOEC) or no effect concentration. The NOEC is the maximum concentration at which the test chemical is not harmful to the tested microorganism compared to controls in a specific test. In addition, the lowest observed effect concentration (LOEC) or minimum threshold concentration (MTC) that is the lowest concentration of the most toxic effect on microorganism compared to controls in a specific test may also be derived. The effects are considered as biological end points since they are vital to the survival, growth, behavior, and perpetuation of a species. These end points vary depending on the type of toxicity test being conducted and the species used [18].

The measurement endpoints detected can be the properties of a microorganism, the components of the test medium, or any substrate, product or metabolite resulting from the activity of the test microorganism [43].

Toxicity test end points may be:

- Growth, regarding cell number, mass production, a nitrogen content of the cell mass, chlorophyll content, reproduction
- Survival or mortality, sometimes immobilization
- Respiration by monitoring O_2 consumption, CO_2 production or measuring the enzyme activities of the respiratory (electron transport) chain, as well as ATP production
- Luminescence
- Other enzyme activities as well as the decrease in the substrate of the enzyme or increase in the product concentration
- Metabolites of biochemical processes
- Gene products such as RNAs and proteins

Biodegradation tests generally use the end points of

- O_2 consumption
- Substrate consumption
- Production of end products such as CO_2 or metabolites from the tested substance.

The inhibitory or toxic effects of a compound can be classified as acute (short-term) or chronic (long-term) effects.

4.3.1 Acute Toxicity Tests

Acute effects are those that arise quickly as a result of short-term exposure to a chemical. The chemical is considered acute toxic if its direct action is to inactivate 50% or more of the exposed population of test organisms in a relatively short period, such as 96 h to 14 days.

Acute toxicity tests are designed to evaluate the relative toxicity of a chemical to selected microorganisms, which are not previously exposed to the inhibitor, upon short-term exposure to various concentrations of the test chemical. These tests may be conducted as a time-dependently (length of time is predetermined to estimate the 24 or 96 h LC50 or the 48 or 96 h EC50) or time-independently (TI) (a duration is continuous until the toxic response manifested) [17, 43].

4.3.2 Chronic Toxicity Tests

Chronic or sub-chronic toxic effects may occur when the chemical produces harmful effects as a result of a single exposure, but more often they are a consequence of repeated or long-term exposures to low levels of persistent chemicals, alone or in combination. Chronic effects also may be lethal or sub-lethal.

Even if a chemical does not have adverse effects on microorganisms in acute toxicity tests, it does not mean that the chemical is not toxic to these species. In addition, chronic toxicity tests allow evaluation of the possible adverse effects of the chemical under conditions of long-term exposure at sub-lethal concentrations. For these reasons, acute and chronic tests should be performed together for accurate results [44].

4.3.3 Biodegradability Tests

Biodegradation is transformation and/or mineralization processes of organic chemical compounds by microorganisms. Basically, there are two types of biodegradation; (1) primary biodegradation is a transformation of organic molecule to another organic chemical compound, and (2) ultimate biodegradation is decomposition of an organic chemical compound into CO_2, H_2O, and salts. Biodegradability of an organic molecule can be determined by standardized tests such as ready biodegradability, inherent biodegradability, and anaerobic biodegradability tests [45].

Mostly, the biodegradability of chemical is determined by the standardized closed bottle tests (CBT) under laboratory conditions. However, the low biomass concentration for inoculation,

high antibiotic concentration in the vessel, and long duration time (28 or 40 days) result in the different biodegradability test results for the same antibiotic [32].

4.4 Aquatic Toxicology Tests

Hundreds of microorganism species are used in standardized and non-standardized toxicity test methods. The suitable test microorganism should be selected according to the aim of the test cause of specific effects that require selective indicator microorganisms such as bacteria, algae, and protozoa. Even though International Standardization Organization (ISO) defined the several species that are used in toxicity tests, just bacteria are evaluated in this chapter [17, 46].

4.4.1 Bacteria

Bacteria are easy to use and rapidly growing organisms, which enable test methods of good reproducibility and are considered to be able to represent their taxa in a wide context. Also, they can be used as toxicological indicator independently of their environmental role. Natural and laboratory strains, mutants, and genetically modified bacteria or mixed cultures are also used to test toxicity of both chemicals and environmental samples. Laboratory strains, *Vibrio fischeri* or *Azomonas agilis*, are used as indicators to detect adverse effects while natural strains are used as endangered species or sensitive key factors of an ecosystem (responsible nitrogen fixation or biodegradation of oil spills and xenobiotics) or as the causes of a hazard. Growth rate, metabolic activity, and metabolic products can be measured as endpoints [17].

Vibrio fischeri is a gram-negative rod-shaped, heterotrophic bacterium found globally in the marine environment. It is predominantly found in symbiosis with various marine animals. It is a fundamental research organism for the examination of microbial bioluminescence, quorum sensing, and bacterial-animal symbiosis. In environmental toxicity testing, it is used test organism, based on the correlation between light emission and toxic chemicals present. The test using *Vibrio fischeri* is standardized (ISO 11348, 2007) and this test organism—due to its wide range sensitivity and easy laboratory use—is applied not only to a marine species, but also as a generic test organism for any environmental sample: water, wastewater, leachate, soil, sediment, solid waste, etc. [17].

Salmonella typhimurium is a gram-negative facultative anaerobic bacterium of the family of Enterobacteriaceae, genus *Salmonella*. It is used in the Ames mutagenicity assay, a short-term bacterial reverse mutation assay using histidine auxotroph *Salmonella*, which carries a mutation in the genes of histidine synthesis [17].

Azomonas agilis is a gram-negative bacterium, motile with peritrichous flagella, found in the water and wastewater. It is capable of fixing atmospheric nitrogen. It is a non-selective organism, used in laboratory bioassays [17].

Other bacteria, first, *Escherichia coli* and the coliforms, as well as other environmental strains from the genus *Pseudomonas*, *Flavobacteria*, *Gammaproteobacteria*, *Bacillus* etc. are often used as test organisms in laboratory bioassays.

4.5 Standardized Test Methods

The mostly used test methods that are standardized by ISO, and OECD to analyze inhibition and toxicity effects of antibiotics on wastewater are listed in Tables 1 and 2. These methods are

Table 1
The most used OECD Toxicity test standards for aquatic toxicology (OECD 2014)

Test guideline	Test name	Principle of the test method
OECD TG 202 (2004)	Daphnia sp., acute immobilization test	In the acute immobilization test depends on a variety of concentrations of the compound investigated struggle varied degrees of toxic effects on the swimming capability of Daphnia. Certain concentrations result in certain percentages of Daphnia being no longer capable of swimming at 24 h but, the test can be extended to 48 h if desired.
OECD TG 209 (2010)	Activated sludge, respiration inhibition test (carbon and ammonium oxidation)	Effects of substances on microorganisms within activated sludge of WWTPs are assessed by measuring their respiration rate
OECD TG 224 (2007)	Determination of the inhibition of the activity of anaerobic bacteria	Predicts the likely effect of a test substance on reduction of gas production from anaerobically digesting sewage sludge
OECD 301 A	DOC Die-Away	That enables the screening of compounds for ready biodegradability in an aerobic aqueous medium by dissolved organic carbon
OECD 301 B	CO_2 evolution (Modified Sturm Test)	That allows the screening of compounds for ready biodegradability in an aerobic aqueous medium by respirometry of CO_2 evaluation
OECD 301 C	MITI (1) (Ministry of International Trade and Industry, Japan)	That enables the screening of compounds for ready biodegradability in an aerobic aqueous medium by respirometry of oxygen consumption
OECD 301 D	Closed bottle	That permits the observing of compounds for ready biodegradability in an aerobic aqueous medium by respirometry of dissolved oxygen
OECD 301 E	Modified OECD screening	That allows the monitoring of compounds for ready biodegradability in an aerobic aqueous medium by dissolved organic carbon
OECD 301 F	Manometric Respirometry	That permits the monitoring of compounds for ready biodegradability in an aerobic aqueous medium by oxygen consumption

Table 2
The most used ISO Toxicity test standards for aquatic toxicology (ISO 2013)

Test guideline	Test purpose
ISO 6341:2012	That uses for the detection of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) - acute toxicity test
ISO 9509:2006	That uses for the detection of toxicity test for assessing the inhibition of nitrification of activated sludge microorganisms
ISO 10706:2000	That uses for the detection of long-term toxicity of substances to Daphnia magna Straus (Cladocera, Crustacea)
ISO 10712:1995	That uses for the detection of the inhibitory effect of surface, ground and wastewater on Pseudomonas putida growth. The test called Pseudomonas putida growth inhibition test (Pseudomonas cell multiplication inhibition test)
ISO 11348-1:2007	That uses for the detection of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test)—Part 1: Method using freshly prepared bacteria
ISO 11348-2:2007	That uses for the detection of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test)—Part 2: Method using liquid-dried bacteria
ISO 11348-3:2007	That uses for the detection of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test)—Part 3: Method using freeze-dried bacteria
ISO 11350:2012	That uses for the detection of the genotoxicity of water and wastewater—Salmonella/microsome fluctuation test (Ames fluctuation test)
ISO 13641-1:2003	That uses for the detection of inhibition of biogas production of anaerobic bacteria—Part 1: General test
ISO 13641-2:2003	That uses for the detection of inhibition of biogas production of anaerobic bacteria—Part 2: Test for low biomass concentrations
ISO 15522:1999	That uses for the detection of the inhibitory effect of water constituents on the growth of activated sludge microorganisms

established for the testing of dissolved chemical substances, surface waters, wastewaters, leachate, and extracts or other waters of agricultural or industrial origin. They can be used easily or in a modified form for the testing of special liquid/solution samples. The aims of testing the adverse effect both of chemicals dissolved in waters and of the waters for different purposes may be fulfilled by the same test design and test organism.

The OECD recommends validated test methods for characterizing the fate and behavior as well as the effect of chemical substances for regulatory purposes. They comprehend tests for the physical-chemical properties of compounds, human health and environmental effects, degradation and accumulation in the environment [47].

The ISO test methods have been developed primarily for testing the effect of environmental water samples, unlike OECD tests that established for pure chemical substances dissolved in water.

In addition to toxicology tests, there are a number of standardized bioassays to determine the biodegradability of a compound and to examine its toxic and inhibitory effects. One of them is biological methane potential (BMP), which is mainly used to evaluate the efficiency of anaerobic digestion for a specific compound. Also, the anaerobic toxicity assay (ATA) has been used to indicate the toxic effects of the compound on methane production. These bioassays have been modified from ISO and ASTM toxicology tests [48].

All of these standardized or non-standardized tests have been used for various antibiotic groups' effects of toxic and inhibitory influence on microorganisms, and their fate and degradation in the WWTPs, and their effects on the environment after treatment.

5 Antibiotics Effects on Microbial Community at WWTPs

Antibiotics can impact microbial communities in sewage systems, negatively affecting the degradation processes and other main processes such as nitrification, phosphate removal, and sulfate reduction [1]. If a wastewater containing antibiotics enters the biological treatment plant, it may inactivate the activated sludge and the living microorganisms which biodegrade the toxic components of the wastewater. If the microflora is inhibited or inactivated, they cannot fulfill their task, the wastewater treatment technology is malfunctioning, and the organic contaminants in the water fail to be degraded. Those antibiotics that cannot be degraded are present in the effluent or are sorbed by the flocks and another form of biofilms of the sewage sludge. However, all antibiotics have different effects on the microbial community according to their chemical properties, biodegradability rate, and concentration in the wastewater.

5.1 β-Lactams

β-lactams consist of amoxicillin, ampicillin, carbenicillin, cloxacillin, penicillin, methicillin, cephalexin, cefprozil, cefuroxime, loracarbef, which have different side chains of β-lactam ring. They are used for wide range of infections, streptococcal infections. It is the most commonly consumed class of the antibiotics for human medicine in most countries [24]. The action mechanism of β-lactams is to bind and inactivate enzymes required for bacterial cell wall synthesis resulting in defective cell walls formation. The activity of glycopeptides polymer units of the cell wall is inhibited by these antibiotics. Eventually this leads to lysis of the cells and death of the organism. Because β-lactam antibiotics are hydrolyzed in the ambient pH and temperature conditions, their concentrations in the environment are low despite their large consumption rate. In addition, β-lactam

rings can easily be affected by heat light, metal ions, oxidizing and reducing agents, nucleophiles, and solvents [10].

Because β-lactams, including penicillins and cephalosporins, have not been detected frequently in WWTPs, corresponding studies on their fate and transformation are limited. In addition, the β-lactam ring is of low stability and can be a breakdown by β-lactamase a widespread enzyme in bacteria or by chemical hydrolysis. As a result, intact penicillin compounds or all β-lactams do not occur in the environment.

There are a lot of studies about biodegradability of penicillins, such as 27% of benzylpenicillin were degraded in 28 days using the Closed Bottle Test (OECD 301 D) [23]. On the other hand, 78–87% of the benzylpenicillin was degraded over 28 days under aerobic conditions which are analyzed by OECD 302 B test. Also, benzylpenicillin ultimate degradation was observed after 60 days under anaerobic conditions by using ISO 11734 test standard [49]. The most frequently detected penicillins were Penicillin G, Penicillin V, Amoxicillin, Ampicillin, Oxacillin, and Cloxacillin at influent of WWTPs. The highest concentrations detected in influent as 13,800 ng/L and in effluent as 2000 ng/L as Penicillin V. For cephalosporins, in total six types were detected with the highest concentration of 64,000 ng/L as cephalexin. The value of 64,000 ng/L is the highest among concentrations of any antibiotic detected in WWTPs and is extremely high for sewage samples. Among these six antibiotics, cephalexin, cloxacillin, cefotaxime, and cefaclor were most frequently detected while cephradine and cefazolin were only detected once in effluent [50, 51].

5.2 Tetracyclines

Tetracyclines have natural and semi-synthetic groups of broad-spectrum polyketide antibiotics which are primarily used to treat infections of the respiratory tract due to *Hemophilus influenzae*, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, sinuses, middle ear, urinary tract, intestines, and gonorrhea by inhibiting the bacterial protein synthesis. However, they are currently used in humans to treat skin disorders such as acne and rosacea, mostly. Although tetracycline antibiotics have been developed for human and agricultural medicine, they are mostly used for veterinary medicine. They consist of an octrahydronaphtacene ring skeleton containing four fused rings with various substitutions positions on the backbone [52, 53]. Even though eight tetracycline antibiotics have been developed for human use, animal agriculture, and poultry industry, there are two main classes as oxytetracycline and chlortetracycline which are widely used in the farm animal industry. Some of the common tetracycline antibiotics include chlortetracycline, oxytetracycline, and tetracycline.

Tetracycline compounds can be found in surface water, groundwater, sediments, soils, and manure at different concentrations that are dependent on several factors including the type of

medium, pH of the solution, and ion content of the matrix [54]. Only five tetracyclines were detected in WWTPs with the highest concentration of Doxycycline in the influent and tetracycline in the effluent as 2210 ng/L and 1420 ng/L respectively [51, 55].

Due to the ability of tetracyclines to form stable complexes with bivalent and trivalent cations, they absorb into sewage sludge with little or no biodegradation during the wastewater treatment. As a result, it is often determined in the soil, sediment, and sludge instead of the aqueous matrix [10, 32].

Tetracyclines were mainly removed through adsorption due to its chemical properties. Tetracycline of 10 ng/L was removed rapidly in activated sludge process via adsorption with the removal efficiency that was up to 95% in 6 h [56]. Moreover, operation parameters such as HRT and SRT are the critical parameters for removal efficiency of tetracyclines at WWTPs. Kim et al. (2005) demonstrated the importance of SRT on removal efficiency of tetracycline. In the study, the removal efficiency of tetracycline was 78.4% at HRT: 7.4 h and SRT: 3 days, whereas it was 86.4% at HRT: 24 h and SRT: 10 days [57].

Cetecioglu et al. (2014) conducted a study to determine biodegradation rate of tetracycline (TET) under various treatment processes by using the modified OECD 311 protocol, and also to examine the effects on the microbial community. According to the result of the study, tetracycline cannot biodegrade under nitrate and sulfate-reducing conditions, but partially (about 46%) biodegraded under methanogenic conditions [33]. Also, sulfate-reducing bacteria were inhibited by TET, while methanogens and archaea were not affected by TET [33]. Nevertheless, another study about biodegradation of tetracycline under anaerobic conditions showed that TET can be degraded up to 91% [58].

The other study about effects of TET on microbial community and activity of nitrifiers was conducted by Matos et al. (2014). The study suggested that 50 μg/L of TET did not significantly affect the removal of organic matter and nitrogen, but the microbial community slightly shifted from 77.8% of Ammonia-oxidizing bacteria (AOB) to 89.8% of *Nitrobacter* spp. [53].

5.3 Macrolides

Macrolides have a lactone ring that is modified with alkyl, ketone, and hydroxyl groups, which are used to treat respiratory systems and soft tissue infections by inhibiting the bacterial protein synthesis. They are primarily bacteriostatic and are particularly effective against gram-positive bacteria and also efficient against anaerobic microorganisms [59]. Their action mechanism is to bind to the 50S subunit of the ribosome, inhibiting bacterial protein synthesis.

Common five macrolide antibiotics include erythromycin, tylosin, spiramycin, roxithromycin with erythromycin—H_2O as a metabolite, are found in both influent and effluent at WWTPs all over the world as a result of continuous and widespread

consumption in both human and veterinary medicine, despite low water solubility. In addition, macrolides can be detected in groundwater and drinking water at high concentrations. Compared to the other five macrolides, oleandomycin was only detected in a WWTP in Australia at relatively low concentrations (20–190 ng/L in influent and 5–150 ng/L in effluent) [50].

As erythromycin can be hydrolyzed very fast, Erythromycin—H_2O can be detected more frequently in the environment rather than parent compounds [1]. Erythromycin—H_2O is the main metabolite of erythromycin which is highly unstable under strong acidic condition. Following erythromycin—H_2O, roxithromycin, clarithromycin, azithromycin, and tylosin were detected at WWTPs. The highest concentration of the macrolides was 10,025 ng/L in influent and 4330 ng/L in effluent as erythromycin—H_2O at WWTP [10, 51].

Cetecioglu et al. (2015) analyzed the acute inhibitory effect of erythromycin on the methanogenic activity in anaerobic digestion of volatile fatty acids. The study showed that 500 mg erythromycin per liter fully inactivated the methanogenic activity [60].

Macrolides cannot eliminate significantly in the biological treatment process. For instance, 3 ng/L of clarithromycin was not removed during 48 h in the activated sludge process [20]. Moreover, macrolides cannot eliminate via adsorption cause of they have low adsorption potential.

5.4 Sulfonamides

Sulfonamides are synthetic broad spectrum antibiotics which are used to treat bacterial and some fungal infections by competitively inhibiting the conversion of p-aminobenzoic to dihydropteroate which is needed by the bacteria for folic acid synthesis. Mainly, sulfonamides have six main groups of antibiotics which are sulfamethoxazole, sulfamethazine, sulfapyridine, sulfadiazine, sulfanilamide, and sulfamethizole characterized by sulfonyl group connected to an amine group. Nevertheless, 16 sulfonamides were detected in WWTPs of Europe, North America, East Asia, and Australia [1, 59].

Sulfonamides are the most common class of antibiotics used in the pharmaceutical residue studies since they are widely used in human and animal medicine and have a good analytical performance for detection. Also, they can be found in the hospital effluent at high concentrations [2, 61].

One of the major antibiotic in the sulfonamide class is sulfamethazine, also known as sulfadimidine, which is widely used to treat food-producing animals. Another important sulfonamide antibiotic is sulfamethoxazole that is used for the treatment of urinary tract infections and effective against *Streptococcus*, *Staphylococcus aureus*, *Escherichia coli*, *Haemophilus influenza*, and oral anaerobes. The highest concentration of sulfamethoxazole was detected in influent as 5597 ng/L and in effluent at 6000 ng/L

at WWTP [62]. Also, a metabolite of sulfamethoxazole, N4-acetylsulfamethoxazole which can be re-transformed to their active parent compound was detected at WWTP [63].

Since the sulfonamides are polar compounds, they can be sorbed to both soil organic matter and soil minerals. Even though sulfonamides degradation occurred during storage in manure lagoons, 40% and 60% of the initial concentrations of sulfamethazine and sulfathiazole, respectively, were found to remain in the manure slurry after 5 weeks of storage [64]. Furthermore, using the OECD Closed Bottle Test, 4% of sulfamethoxazole was degraded in 28 days [23].

The elimination rates of sulfonamides in WWTPs can be very diverse. For example, three sulfonamides (sulfamethazine, sulfamethoxazole, and sulfathiazole) were biodegraded in activated sludge after 3 days without adaptation phase [65]. However, sulfamethoxazole cannot be biodegraded after 40 days according to the OECD 301 D Closed Bottle Test [23].

Ben et al. (2014) conducted a study to investigate fate and behavior via adsorption of sulfonamides by using sulfamethazine (SMN), in the activated sludge process for swine wastewater treatment. They demonstrated that the operation conditions such as MLSS, pH, and SRT significantly affect the adsorption process of SMN into activated sludge. In this study, volatilization and hydrolysis processes did not affect SMN removal. However, SMN completely adsorbed into activated sludge. As a result, the biological activity of activated sludge was inhibited entirely [66].

Under anaerobic conditions, sulfamethoxazole concentrations of up to 40 mg/L in the wastewater were biodegraded, whereas the sulfamethoxazole concentration of 45 mg/L inhibited substrate/COD removal and biogas production [67]. Furthermore, Cetecioglu et al. (2016) conducted research to investigate the effects of sulfamethoxazole on microbial dynamics in anaerobic digestion. The microbial community of anaerobic digestion has maintained system stability even at high sulfamethoxazole concentrations in long-term operation [68].

5.5 Quinolones

Quinolones are polar, mostly atmospheric and low water solubility at neutral pH (6–8) broad spectrum antibiotics that result from their chemical structure that contains two fused rings with a ketone group and carboxylic acid. They are generally used for both gram-negative and gram-positive bacteria, the action mechanism depends on exhibiting bactericidal activity by deactivating of DNA gyrase and topoisomerase which are essential enzymes for the replication of bacterial DNA [14].

Fluoroquinolones are a sub-group of quinolones with a fluorine-substituted central ring. In aquatic systems, fluoroquinolones are susceptible to photodegradation [1].

The quinolones are one of the extensively used antibiotics worldwide. Twelve groups of antibiotics from four generations, two 1st generation ones (pipemidic acid and nalidixic acid), eight 2nd generation ones, and two 4th generation ones (moxifloxacin and gatifloxacin) were determined at high concentrations in hospital effluents and WWTPs influents [10, 32]. Even though these antibiotic classes are most widely detected in WWTP influents and effluents, there are also other quinolone groups such as trimethoprim, thiamphenicol, chloramphenicol, lincomycin, and clindamycin.

The dominant removal pathway for quinolones is secondary treatment via adsorption rather than biodegradation [69]. By this way, 85% of ciprofloxacin, 75% of ofloxacin, and 87% of norfloxacin were removed at WWTPs [56]. In addition, the removal efficiency of COD in activated sludge was decreased while nitrification process was not affected due to the concentration of ciprofloxacin [70].

The half-life of ciprofloxacin in surface water is slightly short, at about 2 h as it is rapidly photodegraded. Using the OECD Closed Bottle Test, ofloxacin was not degraded while about 5% of amoxicillin was found to be degraded in 28 days [23]. A study by Gartiser et al. (2007) showed that between 11 and 63% of amoxicillin was biodegraded in 28 days using the OECD 302 D test while ofloxacin was not degraded [49].

5.6 Others

In addition to these antibiotic classes, there is also a different type of antibiotics such as aminoglycosides, glycopeptides, chloramphenicol, and clindamycin which can be found in influent and effluent of WWTPs, rarely.

Aminoglycosides include a hexose nucleus linkage a glycoside which has two or more amino sugars, resulted by their extremely polar and basic character. Although the use of aminoglycosides is restricted due to side effects and residue in food animals and toxic potential of aminoglycosides, aqueous environment is contaminating with aminoglycosides that are consumed for animal medicine [3].

Glycopeptides are comprised of glycans that are covalently attached to the side chains of an amino acid which are used to treat bacterial infections, particularly against methicillin-resistant *Staphylococcus aureus* (MRSA). Vancomycin and teicoplanin are the most consumed glycopeptides for clinical usage, which cannot eliminate entirely in WWTPs [1, 10].

Besides the studies to investigate a group of antibiotic effects on treatment process and microbial community, there are also several studies that have been carried out to examine the effects of many antibiotic groups on treatment process and microbial community. These studies can be classified into the type of treatment processes such as anaerobic or aerobic degradation processes, nitrification, sulfate reduction, or phosphate removal.

Antibiotics can inhibit the biogas production during anaerobic digestion. However, the inhibition effect may differ depending on the type of antibiotics. For instance, macrolide erythromycin has no inhibitory effect on biogas production, whereas chlortetracycline and chloramphenicol completely inhibit the activity of microorganisms at anaerobic digestion. Moreover, some antibiotics such as ampicillin, novobiocin, penicillin, kanamycin, gentamycin, spectinomycin, streptomycin, tylosin, and tetracycline have partial inhibitory effects such as to hinder the activity of propionic or butyric acid degrading bacteria [59].

Gartiser et al. (2007) conducted a study to assess the anaerobic biodegradability of 9 antibiotics (amoxicillin, benzylpenicillin, chlortetracycline, gentamycin sulfate, monensin sodium salt, nystatin, ofloxacin, sulfamethoxazole, and vancomycin) as well as to determine 16 antibiotics inhibitory effects on anaerobic microorganisms. According to the results, none of 9 antibiotics were significantly biodegraded. Only 20% of benzylpenicillin biodegraded after 40 days. Although none of the tested 16 antibiotics completely inhibited gas production, chlortetracycline, ofloxacin, tetracycline, and 3,5-dichlorophenol inhibited about 90% of biogas production [49].

Long-term effects of antibiotics on nitrification and COD removal as well as their effects on the microbial community were examined by Schmidt et al., 2012. Four different antibiotics ciprofloxacin (CIP), gentamicin (GM), sulfamethoxazole (SMZ) – trimethoprim (TMP), and vancomycin (VA) were used in equal amounts of increasing concentrations from 100 μ/L to 40 mg/L. After 442 days, the long-term impact of antibiotics on wastewater treatment was investigated. According to the results, the COD removal efficiencies were more stable and effective rather than nitrification against different concentrations of antibiotics. Also, nitrification of ammonia stopped by 30 mg/L of the antibiotic mixture, whereas 40 mg/L of antibiotic mixture completely inhibited the nitrification [15].

6 Conclusions

Toxicity and inhibition tests are commonly used and reflect the deactivation and lethal level of each inhibitory/toxic compound. The demand of these tests increases due to development, production, and wide application of chemicals. The main limitations and/or lacks can be listed for the application of these tests on antibiotics:

1. Application principle and test duration of each toxicity inhibition test are different and the result of each test reflects a variety of toxic levels for specific compounds. Thus, more than one test should be applied for same compound to get a comprehensive and representative result.

2. Toxicity inhibition tests are conducted at laboratory scale or pilot scale studies. It should be noted that the response of microbial community generally changes during the scale-up. Therefore, the test may not reflect the exact toxicity/inhibitory level in the real wastewater treatment plants.
3. Each antibiotic class has different characteristics in terms of action mechanism or chemical properties. The inhibition mechanism of each antibiotic class is not well defined in the various wastewater treatment process as well as on biological processes.
4. The various antibiotics are active against different groups of bacteria present in wastewater. Also, behaviors of bacteria are different in pure and mixed cultures. The toxic effects of antibiotic substances on bacteria may change because of the complexity of the inoculum and the various antibiotic spectra of the test compounds.
5. As far as main antibiotic compounds, the toxic and inhibitory action of metabolites and transformation products should be investigated.
6. Antibiotics in wastewater limited the growth potential of microorganism and decreased the microorganism's concentration in the bioreactor as the biological population was able to build antibiotic resistant-bacteria. The effects of antibiotic on wastewater treatment efficiency differ according to the type of antibiotics.

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Chapter 3

Alternative Approaches to Determine the Efficiency of Biomixtures Used for Pesticide Degradation in Biopurification Systems

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Abstract

Biopurification systems were developed for the biological treatment of pesticide-containing wastewaters originated from agricultural production. They are aimed at reducing point-source contamination related to the inadequate handling of pesticides during filling and cleaning of spraying equipment or improper disposal of application residues. These systems contain a biomixture, which comprises their biologically active core where accelerated pesticide degradation takes place. It is a common practice to determine the efficiency of biomixtures by analytical approaches that are focused on the quantification of the pesticides and their subsequent dissipation in time. Nonetheless, the simple removal of original pesticide molecule does not necessarily indicate that the treatment process is ecologically friendly, due to the possible formation of transformation products of high toxicity that are difficult to identify and quantify. This chapter discusses alternative approaches to obtain a more complete scenario regarding biomixture efficiency. These approaches include the determination of the pesticide mineralization using radiolabeled pesticides and ecotoxicological assays to determine the detoxification degree achieved by the matrix. Similarly, as the useful life of biomixtures varies according to the materials employed in its production and the climatic conditions of every region, the authors also suggest a methodology to monitor the performance of biomixtures during their aging process.

Key words Pesticides, Biomixture, Toxicity, Mineralization, Degradation

1 Introduction

1.1 Biopurification Systems for the Removal of Pesticides

Inadequate pesticide handling can cause the contamination of superficial water bodies, groundwater, or elevated volumes of soil. Pesticides can enter water bodies through diffuse or point sources [1]. Influx through diffuse contamination occurs as a consequence of the application of pesticides on the field during agricultural practices. On the contrary, point source contamination arises as a consequence of localized situations where pesticides enter a water body at a restricted number of locations; for example: commercial

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product spills during filling processes, leaks on spraying equipment, residual volumes on pumps and tanks, wastewater derived from internal and external washing of the spraying equipment, among others [2–4]. Even small amounts of spraying solutions have the potential of generating important pollution events [5].

Several physicochemical or biological systems for mitigating the effect of pesticide pollution have been implemented, nevertheless, many of them are costly or require complex technology to be implemented, restricting their use in farms [6]. Due to these limitations, simpler systems, collectively called *pesticide biopurification systems* (BPS) have been implemented. BPS make use of and promote the efficiency of microbial degradation (mainly by bacteria and fungi), and also the adsorptive capacity of its components, in order to restrict the impact that pesticides could have in the environment.

BPS were first implemented in Sweden in the 1990s as simple and cheap constructions built to contain and degrade pesticide spills in agricultural farms [7], but also residues derived from cleaning the spraying equipment [4].

BPS are excavations or containers filled with a biologically-active matrix called biomixture, composed of soil, a lignocellulosic substrate, and a component with a high humic content mixed at different proportions [7, 8]. The efficiency of any BPS is based on the ability of the biomixture to effectively degrade and retain high loads of pesticides being discarded on the system [8]. Therefore, the adsorption and microbial degradation capabilities of the biomixture play a fundamental role to attain an adequate performance.

The different components of the biomixture carry out different functions in the BPS. Soil contributes to the adsorption capacity of the system and provides microorganisms with pesticide degradation abilities. It has been shown that this contribution is greater if the soil has been previously exposed to the pesticide of interest [9]. Lignocellulosic substrates act as a colonization medium for ligninolytic fungi and potentiate their enzymatic degradation activity towards organic contaminants. Moreover, the liberation of simpler molecules from lignin degradation serves as a source of nutrients for the rest of microorganisms present in the biomixture. The humic component contributes to moisture control, adsorption capacity and pesticide degradation [6, 7].

BPS have been implemented in more than 25 countries, in many of which the specific composition and configuration of the systems has been modified in order to adapt them to local climate conditions, available materials and legislation [6, 8].

The first BPS configurations used peat as the humic component, nonetheless, during the BPS adaptation process to other latitudes, the use of urban or garden compost has been widely adopted [10]. In general, compared to peat, compost is characterized for having a lower carbon content, higher levels of

macronutrients (N, P, K), and a neutral to alkaline pH capable of maintaining a metabolically active microbial community [11]. In contrast, peat has higher water retaining capacity, a considerably lower density, a lower pH, higher carbon content, lower nitrogen content, and in general, biomixtures with this component are unsuitable for stimulating a metabolically active microbial community, but instead they promote cometabolic transformations typically associated with white-rot fungi [8]. Furthermore, peat has a higher pesticide adsorption capacity, while, in general, compost-based biomixtures show a higher degradation capacity [12–15].

Regarding their physicochemical characteristics, the different types of soils used in biomixture preparation seem to have minor effects on the efficiency of pesticide degradation in the matrix. Nonetheless, care should be taken not to use soils with very high clay contents, because their high adsorption capacity could restrict the bioavailability of the pesticides for microorganisms, thus leading to reduced biodegradation [16].

The list of commonly used lignocellulosic substrates includes sugarcane bagasse, wheat straw, sawdust, and coconut fiber, among others. These materials are added to the biomixture in a percentage of up to 50% (v/v), even though lower percentages have been assayed with success [17, 18], and particular biomixture compositions have been optimized for the removal of specific pesticides [19, 20].

More studies that evaluate different modifications to the original BPS biomixture design are needed; for example, the effect of changing straw for other lignocellulosic materials and replacing peat by compost on the biomixture must be further assessed, because the resulting change in pH can increase the mobility of some pesticides and favor other processes different from the non-specific fungal activities [7].

1.2 Monitoring the Biomixture Efficiency

Bioremediation approaches aim to detoxify contaminated matrices based on biological transformations; however, the compound transformation alone does not represent a guarantee that detoxification takes place in the biomixture, given that unknown transformation products could be produced in the degradation processes. Sometimes, these transformation products can be as toxic or even more toxic than the parent compound, thus translating into failed bioremediation process.

The usual determination of biomixture performance is based on the quantification of the pesticide removal by means of HPLC or GC coupled to different techniques of mass spectrometry. Nonetheless, the metabolic processes taking place within the matrix result in the formation of diverse transformation products or metabolites. The determination of such metabolites in situ is a challenging and costly task, given that their nature is usually unknown, and their concentrations are usually extremely low. Even though some metabolites may be commercially available to be employed as

standards, the diversity of metabolites is too wide to be completely covered by an analytical chemistry approach. Data on metabolites production in BPS is limited so far. The formation of toxic metabolites such as 3-hydroxycarbofuran or 3-ketocarbofuran from carbofuran [21] or 3,5,6-trichloropyridynol (TCP) from chlorpyrifos hydrolysis [17, 22] has been described in biomixtures; nonetheless, in some cases these products are also eliminated in the matrix. Lower accumulation of transformation products derived from chlorpyrifos, terbuthylazine, and metribuzin was found in biomixtures with respect to soil [23]. Similarly, the same transformation products have been detected in soil and biomixtures for other pesticides such as isoproturon [24].

In the case of BPS, the demonstration of pesticide mineralization is an indicator that the accumulation of potentially toxic metabolites is not taking place in the biomixture. A mineralization process indicates that the agrochemical is completely oxidized to inorganic compounds, that is, CO_2 and H_2O (under aerobic conditions), thus preventing the accumulation of organic transformation products. Obviously, the mineralization is a much slower process than transformation, and before mineralization occurs, intermediate toxic products could be formed; nonetheless, achieving mineralization suggests that such products would be eventually removed from the biomixture. Numerous works describe the mineralization of pesticides in soil [25–28], nonetheless, mineralization of pesticides in BPS is scarcely reported; this process has been demonstrated in biomixtures for pesticides such as chlorpyrifos [17], carbofuran [21] or linuron [9, 29]. A list of some reports dealing with the mineralization of pesticides in biomixtures can be seen in Table 1.

Given that the main goal of BPS is the depuration and detoxification of pesticide-containing wastewaters [23], the determination of residual toxicity of the matrix after the treatment constitutes an easy way to globally estimate the eco-friendliness of the process. As described above, the complex transformation process that results in the formation of unknown metabolites within the biomixtures is linked to the risk of production of highly toxic compounds. In this respect, the monitoring of ecotoxicological changes during the degradation process permits an indirect determination of the occurrence of such toxic metabolites, even though they are not identified or quantified. The ecotoxicological assessment of biomixture performance should include tests with bioindicators from different levels of the trophic chain in order to obtain a complete panorama of the possible toxicological effects. So far, studies on BPS have seldom included ecotoxicological tests to monitor detoxification of biomixtures. Several works dealing with the degradation of the insecticide/nematicide carbofuran alone [20, 21], or in combination with chlorpyrifos [31], reported an almost complete toxicity removal based on acute immobilization tests on *Daphnia magna*.

Table 1
Selected reports on the determination of pesticide mineralization in biomixtures

Pesticide	Biomixture	Use of radiolabeled pesticides	Reference
2,4-dichlorophenoxiacetic acid dimethylamine salt	Soil-manure compost-wheat straw (25:25:50)	No	[30]
Bentazone	Soil-peat-straw; compost-citrus peel; compost-straw (different proportions)	Yes	[12]
Carbofuran	Soil-compost-coconut fiber (25:25:50)	Yes	[21, 31]
	Soil-compost-rice husk; soil-peat-rice husk (25:25:50) (fungal bioaugmentation)	Yes	[32]
Chlorpyrifos	Soil-peat-straw; compost-citrus peel; compost-straw (different proportions)	Yes	[17]
	Soil-compost-coconut fiber (25:25:50)	Yes	[31]
	Soil-compost-coconut fiber (42:13:45)	Yes	[33]
Isoproturon	Soil-peat-straw; compost-citrus peel; compost-straw (different proportions)	Yes	[12]
Linuron	Soil-peat-straw (different proportions)	Yes	[9, 29]

Nonetheless, chronic tests on *D. magna* and fish such as *Oreochromis aureus* demonstrated that despite acute toxicity was mostly removed, chronic effects were still produced by sublethal pesticide concentrations remaining in the biomixtures [34]. Similarly, failure in the elimination of the toxicity toward *D. magna* was observed in the simultaneous treatment of nine pesticides with different activities; on the contrary, a successful decrease in the phytotoxicity, measured by germination tests with *Lactuca sativa* seeds, was detected and regarded to be mostly due to the removal of the herbicides in the matrix [35]. Such results highlight the pertinence of including several bioindicators, and acute as well as chronic assays when applying ecotoxicological tests to monitor the performance of biomixtures. Table 2 lists several works that included ecotoxicological monitoring of pesticide degradation in biomixtures.

1.3 Overview of the Monitoring Procedures

The analysis of pesticide mineralization is based on the use of ^{14}C–radiolabeled pesticides. When pesticide mineralization is taking place in the biomixture, CO_2 will be produced; however, normally this will be indistinguishable from the CO_2 produced due to microbial respiration in the matrix by the use of other substrates. When using a ^{14}C–radiolabeled pesticide, its mineralization will yield $^{14}CO_2$, which can be distinguished by the radioisotope activity. The test is based on capturing the $^{14}CO_2$ (and also non-radiolabeled CO_2) in a KOH trap, in which $K_2{}^{14}CO_3$ is formed.

Table 2
Ecotoxicological studies during the removal of pesticides in biomixtures

Pesticide	Biomixture	Bioindicator	Reference
Carbofuran	Soil-peat-several lignocellulosic residues; soil-compost-several lignocellulosic residues (25:25:50)	*D. magna* (acute)	[21]
	Soil-compost-coconut fiber (different proportions)	*D. magna* (acute)	[19]
	Soil-compost-rice husk (25:25:50)	*D. magna* (chronic); *Oreochromis aureus* (chronic)	[34]
	Soil-compost-rice husk (different proportions)	*D. magna* (acute)	[20]
Carbofuran + chlorpyrifos	Soil-compost-coconut fiber (25:25:50)	*D. magna* (acute)	[31]
Mixture of carbamates: aldicarb, carbofuran, methiocarb, methomyl	Soil-compost-rice husk (27:43:30)	*D. magna* (acute)	[36]
Mixture of: atrazine, ametryn, linuron, imidacloprid, thiamethoxam, carbendazim, metalaxyl, tebuconazole, triadimenol	Soil-compost-coconut fiber (42:13:45)	*D. magna* (acute); *L. sativa*	[35]

The subsequent analysis of the KOH solution by liquid scintillation permits quantifying the activity corresponding to the $^{14}CO_2$ radioisotope. Cumulative data of $^{14}CO_2$ production (expressed as activity) allows the determination of the amount of pesticide mineralized, with respect to the initial activity of the ^{14}C–pesticide added in the biomixture.

To perform ecotoxicological analysis in BPS, the biomixture samples are subjected to a liquid extraction that simulates the production of leachates. As BPS are somewhat confined depending on the configuration, the higher risk related to exposure to the environment is the release of biomixture leachates [37, 38]. The liquid extract or elutriate is then used as the matrix that interacts with the bioindicators according to each specific test. In this chapter the acute test on *D. magna* immobilization and the seed germination test on *L. sativa* are covered; though the tests are based on standard protocols [39, 40], some variations are indicated.

Finally, maturity or aging of biomixtures is an important factor that affects their performance [41]. Aging results in the

degradation of the organic matter in the matrix, which has been linked to slower removal of pesticides and increased accumulation of transformation products due to reduced microbial activity [42]; on the contrary, aging may also enhance biomixture sorption capacity [43], a recommended feature to reduce the risk of leachate formation. In this respect, the estimation of the useful life for BPS has not been clearly established. Castillo et al. [7] suggest that a BPS can be used for as much as 6 years, and that the volume losses of biomixture should be simply restored in the system. Similarly, some authors indicate that the moment at which the C/N ratio in the biomixture matches that of soil, the matrix should be replaced. However, these considerations are determined at latitudes with seasonal weather, where hard winter periods may decelerate microbial activity and therefore result in longer useful lives than those obtained, for instance, in the tropics, where the temperatures permit a faster consumption of the organic matter contained in the biomixture, thus resulting in shorter useful periods. For these reasons, a simple test to monitor the efficiency of the biomixture during its aging is described in this chapter. The test is based on the determination of the removal of a target pesticide over a short period, which is performed sequentially during the use of a biomixture. A decrease in the removal of the pesticide through time will reveal a loss on the degrading capacity of the matrix. The test requires previous knowledge on the estimated half-life of the pesticide in the biomixture. The extent of removal efficiency loss can be used to determine the useful life of the biomixture for the specific target pesticide, and will indicate the moment to substitute part of the organic matter or the complete disposal of the matrix.

2 Materials

2.1 Mineralization of ^{14}C–Pesticides

1. Biometer flasks (*see* Fig. 1).
2. Solutions: KOH solution (0.1 M); Ultima Gold™ liquid scintillation cocktail.
3. Pesticides: ^{14}C–radiolabeled pesticides and commercial formulations of the same pesticides.
4. Liquid scintillation counter (LS6000SC, Beckman Instruments Inc.).
5. High performance glass vials for liquid scintillation counting.
6. Micropipettes and the appropriate tips; 60 mL syringes.
7. Precision and analytical balances; incubator at adjustable temperature.

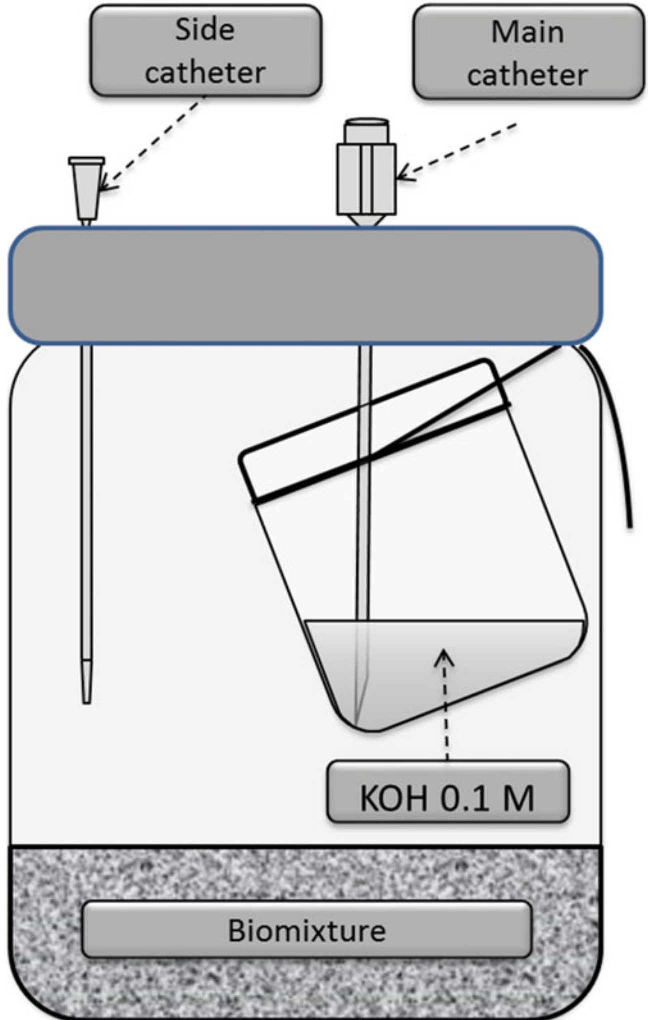

Fig. 1 Biometer flask used to trap $^{14}CO_2$ in the assays of ^{14}C–pesticide mineralization (Adapted from [44])

2.2 Ecotoxicological Assays

2.2.1 Elutriate Preparation

1. 100 g biomixture sample: each sample taken must be at least of 100 g to obtain enough elutriate for ecotoxicological assays.
2. 400 mL moderately hard reconstituted water: 10 mL solution A (6 g L^{-1} $MgSO_4$; 9.6 g L^{-1} $NaHCO_3$; 0.4 g L^{-1} KCl) plus 30 mL solution B (2 g L^{-1} $CaSO_4 \cdot 2H_2O$), per liter of distilled water.
3. Eight conical centrifuge tubes (50 mL).
4. Cloth filter.

2.2.2 Acute Test on *D. magna*

1. At least 230 neonates of *D. magna* (less than 24 h).
2. 18 glass vessels (30 mL).

3. Moderately hard reconstituted water.
4. Plastic pipettes (1 mL).
5. Stereoscope.

2.2.3 Germination Test on L. sativa

1. At least 120 *L. sativa* seeds without chemical treatment.
2. Sodium hypochlorite: 3–4% w/v.
3. Distilled water and moderately hard reconstituted water.
4. Six disposable petri dishes (90 mm diameter).
5. Six filter paper (minimum 84 g m^{-2}).
6. Tweezers; parafilm; ruler; 2HB pencil; towel paper.

2.3 Estimation of Pesticide Removal Efficiency During Biomixture Aging

1. 500 g of biomixture.
2. 1.0 L graduated glass beaker.
3. Commercial formulation of the desired pesticide.
4. Adequate equipment and methodology for quantification of the desired pesticide.
5. Purified water (distilled or deionized).
6. Laboratory balance.
7. 10 or 25 °C incubator.

3 Methods

3.1 Mineralization of ^{14}C–Pesticides

3.1.1 Setup

1. Prepare the biometer flasks as described in Fig. 1: use a 400 mL glass jar with a metal lid and suspend a 50 mL glass flask by copper wires inside the largest jars; place two catheters in the upper section (lid) of the jar; the main catheter will be directly placed inside the small flask and will be used to withdraw the KOH samples and to add fresh KOH solution. The side catheter will be opened during the operations that involve the use of the main catheter. Use triplicate systems, that is, prepare three biometer flasks for each biomixture (or condition) to be tested (*see* **Note 1**).
2. Place 50 g of the biomixture inside each biometer flask; previously adjust the water content to around 60–70% water holding capacity (WHC).
3. Spike the biomixture with the target pesticide at a biomixture relevant concentration (around 10–50 mg kg^{-1}). Use commercial formulations and prepare intermediate solutions in order to add aliquots of around 1–2 mL. Homogenize the matrix; use small wood sticks and leave then inside the flasks.
4. Spike the biomixture with the ^{14}C–radiolabeled pesticide, in order to obtain an activity in the range of 2500–5000 dpm g^{-1}

in the biomixture (*see* **Note 2**). Prepare intermediate solutions in order to add aliquots of around 500–1000 μL in the biomixture. Homogenize the matrix; use the wood sticks as described above.

5. Prepare triplicate blanks: biometer flasks containing the biomixture plus the non-radiolabeled pesticide.
6. Close the lid of every biometer flask and using the catheter, add 10 mL of the KOH solution (0.1 M). Close the catheters using their caps.
7. Register the total weight of each biometer flask to add water in case of water losses during the assay.
8. Place the biometer flasks in an incubator at 25 °C (or the desired temperature at which the biomixture will operate), for a period of at least 4 weeks.

3.1.2 Sampling

1. At each sampling point, remove the cap from the catheters and using a syringe on the main catheter, withdraw the KOH solution contained in the internal flask and place it in a labeled vial. Use a separate syringe for this step, as it will be contaminated with radiolabeled compounds.
2. Using another syringe and the main catheter, add 10 mL of fresh KOH solution (0.1 M) in the internal flask.
3. Use the side catheter to inject around 200 mL of air with a syringe. Place the caps on the catheters.
4. Weigh each biometer flask and replace the losses with distilled water if necessary.
5. Incubate the biometer flasks until the next sampling point.

3.1.3 ^{14}C–Activity Analysis and Data Analysis

1. Transfer 2 mL from each KOH sample into a glass vial for liquid scintillation counting and add 8 mL of Ultima Gold™ liquid scintillation cocktail.
2. Measure the ^{14}C–activity in the liquid scintillation analyzer; set counting time in 5 min and the isotope in ^{14}C.
3. Subtract the activity recorded in the blanks to that determined for the reaction systems.
4. Calculate the percentage of the applied dose (in terms of activity) that was converted to $^{14}CO_2$ with respect to total initial activity spiked in the matrix.
5. Plot the data of cumulative percentage of $^{14}CO_2$ produced as a function of time to perform kinetics analysis of mineralization (*see* **Note 3**).

3.2 Ecotoxicological Assays

3.2.1 Elutriate Preparation

1. Prepare elutriates by mixing the sample and moderately hard reconstituted water in a ratio 1: 4 (g mL^{-1}). Eight centrifuged tubes with this mixture produces enough elutriate for the tests.
2. Shake mechanically the mixture for 1 h in a bench mixer (at 2500 rpm) and centrifuge for 10 min at 2200 × g; use the resultant supernatant as elutriate. Filter the elutriate to remove particles that may interfere with the test.
3. Conserve at 4 °C if not used immediately.

3.2.2 Acute Test on D. magna

1. Test five different concentrations of the elutriate spaced apart by a dilution factor of 2; the original elutriate represents a 100% concentration. Use moderately hard reconstituted water as an eluent to prepare the dilutions (*see* **Note 4**).
2. Using a plastic pipette, transfer 1 mL of moderately hard reconstituted water containing 10 daphnid neonates into each glass vessel. Then expose the neonates to 20 mL of the proper dilution of the elutriate, in darkness at (22 ± 1) °C. Perform this step in triplicates for each dilution of the elutriate.
3. Register mortality/immobility at 24 and 48 h according to the visualization of the daphnids in the vessels (*see* **Notes 5** and **6**). Use mortality/immobility of the daphnids (as the sum of affected organisms in all the replicates) in each dilution to calculate the half maximal effective concentration (EC_{50}) using the TOXCALC—Toxicity Data Analysis Software (Tidepool Scientific Software, CA, USA) or another similar software. Express toxicity results as toxicity units (TU), calculated according to the expression: $TU = (EC_{50})^{-1} \cdot 100$ (*see* **Note 7**).

3.2.3 Germination Test on L. sativa

1. Place at least 100 seeds of the same size and color and without fractures for 10 min in the sodium hypochlorite solution for disinfection. The amount of hypochlorite solution should be enough to cover the seeds.
2. Discard the sodium hypochlorite and wash the disinfected seeds three times with distilled water. At the end, place the seeds in paper towel; discard seeds that exhibit signs of damage, different color or different size. At least 70 seeds must be kept for testing.
3. Place filter paper inside the petri dishes to cover the bottom surface and transfer 5 mL of the elutriate into each petri dish. Then, using tweezers, place 10 seeds on the filter paper, distanced 1 cm apart from each other. Incubate in darkness at (22 ± 1) °C. Use moderately hard reconstituted water as negative control. Perform the test in triplicates (*see* **Note 8**).
4. After 6 days, register germination and root elongation (*see* **Note 9**) to calculate relative seed germination (SG), relative

root elongation (RE), and germination index (GI), using the following equations (*see* **Note 10**).

$$SG = \frac{\text{seeds germinated}}{\text{seeds germinated in control}} \times 100$$

$$RE = \frac{\text{mean root length}}{\text{mean root lenght in control}} \times 100$$

$$GI = \frac{(SG) \times (RE)}{100}$$

3.3 Estimation of Pesticide Removal Efficiency During Biomixture Aging

1. Before the experimental setup, follow the previous considerations indicated in **Notes 11–13**.
2. Place approximately 500 g of the biomixture in a 1.0 L graduated glass beaker.
3. Spike the biomixture with the selected commercial formulation of the pesticide dissolved in purified water in order to achieve both the desired concentration of active ingredient and the moisture of the biomixture that were determined beforehand.
4. Thoroughly homogenize the spiked biomixture.
5. Weigh the entire system and keep a record of this parameter. Repeat the weighing process on a weekly basis and maintain constant moisture in the biomixture by adding an amount of purified water equivalent to the mass lost by evaporation during the previous week (*see* **Note 14**). Also record the initial volume of the biomixture in the beaker.
6. Take triplicate samples from different sections of the homogenized biomixture in order to determine the initial pesticide concentration by any available methodology.
7. Incubate the system in the dark for a period of time equivalent to one half-life of the pesticide at 10 or 25 °C; the temperature must be chosen depending on the weather conditions where the actual BPS will be located (this temperature should also match the one used for the previous half-life determination).
8. After this period, thoroughly homogenize the biomixture and take additional triplicate samples from different sections of the homogenized biomixture to determine the percentage of the initial pesticide removed.
9. Incubate as before for a period equivalent to 10 half-lives of the pesticide in the biomixture.
10. Repeat **steps 3–9** until the pesticide degradation capacity decreases to such an extent so as to have a statistically significant difference with respect to the average of all the previous determinations recorded in the assay. If a trend of slow

efficiency decrease through time is observed, the last value obtained can be compared to the average of the first 3–5 determinations, instead of the whole set; this will allow for gradual efficiency decrease not to go undetected.

11. This procedure will reveal the period of time in which a loss on the pesticide-degrading capacity occurs in the matrix and therefore an approximation of the maximum expected useful life of the biomixture for the specific target pesticide (*see* **Note 15**).

4 Notes

1. Standard commercial biometer flasks may be used if available (Bellco Glass Inc., Thomas Scientific).
2. Estimation of mineralization depends on the positions of the radiolabeled C atoms within the pesticide molecule. Radiolabeling of C atoms at positions that are easily split off from the pesticide molecule will result in faster mineralization estimations; on the contrary, radiolabeling of C atoms in more recalcitrant fragments of the molecule (i.e., aromatic rings) will produce slower mineralization estimations, given that their transformation will probably require slower reactions or more reactions. The ideal scenario would be to use pesticide standards radiolabeled in every C atom; nonetheless, they are not easily produced and their availability is limited. In general, the use of standards radiolabeled in molecule positions which are more difficult to transform, will provide a better estimation of the mineralization process of the molecule as a whole.
3. For the kinetics analysis, a first approach usually considered is the first order reaction of decay in the activity of the ^{14}C–pesticide as it is transformed in $^{14}CO_2$ (in case no clear lag phase is detected for the mineralization process); in this case plot the remaining percentage of activity of the ^{14}C–pesticide versus time. In case of lag phases in the process, the description of the kinetics can be implemented with sigmoid models such as logistic, Gompertz, or Weibull, among others. Always use different models in order to determine the one that better describes the data collected.
4. To determine the test concentrations (i.e., the respective dilutions of the elutriate) it is necessary to perform a preliminary test, using three concentrations (dilutions) and five daphnid neonates in 10 mL of each concentration (dilution). Use triplicates. If the toxicity of the sample is unknown the first dilutions to be tested in the preliminary assay should be 100%, 25%, and 6.25%. If a high toxicity is suspected in the sample, lower concentrations (dilutions) should be tested in

the preliminary assay. If no effects are observed in the 100% elutriate (original elutriate), it is not necessary to conduct a complete test.

5. For a better visualization of mortality/immobility it is recommended to use a white background and to shake in an orbital manner the vials to reactivate the movement of the organisms that are located at the bottom. In case of a doubt, a stereoscope should be used to check the absence of heart rhythm in neonates. To avoid spills during observation, it is necessary to use vials with lid or instead seal the vessels with parafilm. Also, after visualization, check that all organisms are floating in the solution (i.e., they are not stuck to the walls of the vial).
6. For a test to be valid, no more than 10% of the neonates can be immobilized in the control group (no more than one organism in each replicate); similarly, dissolved oxygen concentration at the end of the test should be $\geq$3 mg L^{-1} in control and test vessels.
7. Probit analysis is the preferred choice to report EC_{50}; if reasonable values for confidence intervals are not obtained, use the Binomial analysis.
8. To ensure adequate moisture in each petri dish, they should be sealed with parafilm; in addition, the filter paper should be slightly larger than the bottom plate.
9. To facilitate the measurement of the length of the root, stretch the root with the help of the tip of the pencil so that it does not break.
10. Record variations on the germination profiles, such as secondary root growth, density of root hairs and opening of cotyledons, as they could represent stress responses due to the toxicity of the elutriates.
11. The moisture content of the biomixture to be used in the assay should be adjusted to be similar to the one at which it is used in the field. Moisture content of biomixtures can be determined by using most methodologies usually employed to measure soil moisture. A simple oven-drying method has been previously described in the literature [45].
12. The initial concentration of pesticide in the biomixture used to carry out the assays must be selected, aiming to match the concentrations that can be found in a BPS. This should be determined taking into consideration the specific pesticide that will be used, the size of the BPS, and the guidelines regarding application frequency and dosage. As BPS are expected to receive much higher doses of pesticides than soil, concentrations between 10 and 50 mg a.i. kg^{-1} biomixture are usually calculated for this parameter.

13. An estimate of the dissipation half-life (DT_{50}) of the pesticide in the previously unexposed biomixture is required. If no data are available, a laboratory removal half-life determination may be performed beforehand. General guidelines to carry out this process are publically available [46].
14. For this calculation remember not to take into consideration the mass of biomixture removed during sampling for pesticide quantification.
15. In the actual BPS, when this period of time passes you may either decide to carry out the complete disposal of the biomixture or to substitute part of the lignocellulosic substrate in order to replenish the depleted carbon sources. The amount of lignocellulosic substrate added should be equal to the loss of volume in the system.

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Chapter 4

Bioremediation of Methomyl by *Escherichia coli*

Amritha G. Kulkarni and Basappa B. Kaliwal

Abstract

Methomyl belonging to N-methyl carbamate group of insecticide classified as a toxic, hazardous and restricted use pesticide by the World Health Organization (WHO), European Commission (EC), and Environmental Protection Act (EPA), is the most commonly used pesticide on fruits and vegetables. The present study is, therefore, aimed to study the potentiality of *Escherichia coli* in the degradation of methomyl and further determine the role of plasmid in bioremediation. The organism was inoculated in a synthetic medium containing methomyl (10^{-3}–10^{-7} M) for 24, 48, 72, and 96 h and subjected to HPLC analysis where methomyl degradation (%) was observed. The present study on HPLC analysis at regular intervals of 24 h of *Escherichia coli* in the synthetic medium containing methomyl (10^{-3}–10^{-7} M) incubated for 96 h revealed that there was a significant decrease in the methomyl content in all the treated groups when compared with the initial control, thus offering a promising strategy toward bioremediation. The plasmid isolation and curing study confirmed that the genes of both the plasmid and the main chromosome of *Escherichia coli* are involved in the methomyl degradation. The evolution and spread of pesticide-degrading genes may have a beneficial effect by removing toxic pollutants from the environment. Such organisms can be a source of resistance genes for cloning purpose which have potential use in biotechnology such as the manufacture of biosensors and bioremediation processes.

Key words Bioremediation, Methomyl, *Escherichia coli*, Plasmid

1 Introduction

A wide variety of synthetic chemicals have found their way into the ecosystem as a consequence of industrial activities, agricultural applications, and use in domestic purpose [1]. Pesticides are organic compounds manufactured and used for pest control. When pesticides are dispersed in the environment, they become pollutants, with ecological effects that require remediation. Environmental pollution is caused by both excessive and continuous use of pesticides, and begins when these compounds enter the environment by various means (accidental spills, direct application, residues from cleaning of containers, state of equipment used, and methods used to apply the products). The quality of soils, ground water, inland and coastal waters, and air are all affected by pesticide contamination [2].

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The microbial degradation of hazardous waste offers a promising strategy by which such chemicals may be detoxified [3, 4]. Methomyl belongs to a class of compounds known as oxime carbamates, and it is widely used for the control of insects and nematode pests by inhibiting the enzyme acetylcholinesterase which hydrolyzes the neurotransmitter acetylcholine. The IUPAC name of methomyl is S-methyl N- (methylcarbamoyloxy) thioacetimidate [5]. The WHO (World Health Organization), EPA (Environment Protection Agency), and ECC (European Chemical Classification) classify methomyl as a very toxic and hazardous pesticide. Methomyl is highly soluble in water, and since the sorption affinity of soils for this pollutant is rather low, it can easily cause contamination of both ground and surface water resources [6]. The excessive use of pesticides leads to an accumulation of a huge amount of residues in the environment, thereby posing a substantial health hazard for the current and future generations due to uptake and accumulation of these toxic compounds in the food chain and drinking water [7]. The residual pesticide comes in contact with water causing surface/ ground water pollution leading to the toxicity of biotic environment. Therefore, bioremediation techniques for the treatment of pesticides are of paramount importance [8, 9]. Bioremediation constitutes an attractive alternative to physico-chemical methods of remediation, as it is less expensive and can selectively achieve complete destruction of organic pollutants [10]. Microorganisms are thought to play an important role in the removal and detoxification of these toxicants from the environment.

Degradation of pesticides is usually beneficial, since the reactions that destroy pesticides, convert most pesticide residues in the environment to inactive, less toxic, harmless compounds [11]. The prokaryotes compose an interesting group of microorganisms, which can be used as instruments of scientific investigation of them. *Escherichia coli* is an important biotechnological tool, which makes it possible to obtain important parameters for the metabolic and genetic characterization of cells of more complex organisms [6, 12]. Five bacterial isolates (*Pseudomonas aeruginosa, Klebsiella Sp., Escherichia coli, Bacillus Sp. and Corynebacterium*) isolated from brinjal cultivated field were found to degrade cypermethrin [13]. Numerous strains capable of degrading compounds such as 2,4-D and monocrotophus have been isolated from various environments and have been found to be distributed over many different phylogenetic groups [14, 15]. The *Burkholderia* sp. Strain BBK_9 play a significant role as a biological candidate for cleaning up of the contaminated pollutants. The analysis and toxicity of methomyl and ametryn after biodegradation was performed using an enriched mixture of activated sludge collected from domestic waste water treatment plan [16]. The microbial diversity may have been markedly changed following pesticide used despite unaltered metabolism and such change may affect the soil fertility [17]. The

ability of *Escherichia coli* to grow to far higher densities than *P. diminuta* and *Flavobacterium* enables its use in large-scale detoxification processes. Recombinant DNA technology is a relatively new and fast growing area where the bacterial plasmids are being relatively used as cloning vehicles, because of its small size and relaxed mode of replication, resulting in multiple copies in a cell. Whole cell microorganisms have been widely employed to biodegrade environmental toxic materials. Wild-type microorganisms have limitations, such as low growth rate, low cell density, and feed-back inhibition of metabolism [18, 19]. A significantly improved, recombinant *Escherichia coli* has been developed to degrade the toxic organophosphorus compound, Paraoxon, to non-toxic materials; therefore, engineered recombinant microorganisms have been of great interest [20].

Pesticide degrading genes in microbes have been found to be located on plasmids, transposons, and/ or on chromosomes. Some microbial strains possess genetic determinants that confer resistance. In bacteria, these determinants are often found on plasmids, which have facilitated their study at the molecular level [21]. Bacterial plasmid plays a role in the degradation of the pesticide [22]. Many of the micro-organisms capable of metabolizing synthetic organic compounds like pesticides, harbor large degradative plasmids [23]. Kumar et al. [24] have reviewed microbial degradation of pesticides with special emphasis on the catabolic genes in relation to pesticide degradation. The involvement of naturally occurring plasmids in the degradation of carbaryl has been extensively documented [25]. Such plasmids and chromosomes increase the biochemical versatility of the host bacterium, extending the range of complex organic compounds used as sole sources of carbon and energy [26]. Therefore, the present investigation was undertaken to study the bacteria for its capability to degrade methomyl. Further, attempt was also made to determine the role of plasmid or chromosome involved in the biodegradation of methomyl.

2 Materials and Methods

2.1 Preparation of Stock Solution of Methomyl

The sample of methomyl (Lannate®) used in the experiment was commercial insecticide supplied by E.I. Dupont India Pvt. Ltd., Haryana obtained from the local company's market containing 40% (w/w). The stock solution of 0.1 M of methomyl was prepared in distilled water and sterilized separately. This 0.1 M stock solution was further diluted to give different required molar final concentrations.

2.2 Maintenance and Propagation of Culture

The organism *Escherichia coli* was procured from NCL, Pune, was maintained at 4 °C on nutrient agar, and was subcultured every fortnight. Synthetic medium (S-medium) formulated by Lackey and White [27] was prepared for toxicity texting. Pre-inoculum

was prepared by inoculating a loopful of bacteria from the overnight incubated nutrient agar slant cultures on a 100 mL sterilized synthetic medium and incubated for 18–24 h at 37 °C under static conditions depending on the exponential phases of bacteria under test. The strain was subjected to high performance liquid chromatography (HPLC) and the percentage degradation was assayed.

2.3 Determination of Methomyl (%) Using HPLC

The HPLC analysis was carried out as per Tamimi [28]. High performance liquid chromatography (HPLC) analysis was performed at USIC Department, Karnatak University Dharwad with a Shimadzu HPLC system. The analytical column used was C-18 (150 mm × 3 mm, 3 μm). The flow rate was 0.5 mL min^{-1} and the injection volume was 20 μL. the isocratic elution conditions were acetonitrile and water. The wavelength for detection was 231 nm.

2.4 Plasmid Isolation and Curing of *Escherichia coli*

Plasmid DNA was isolated and cured by following the method described by Brown [29].

The LD-50 values were determined using a curing agent and then the cultures were subjected to plasmid curing. The *Escherichia coli* culture was grown in different concentrations of acryflavin (0–50 μg/mL) for 24 h in nutrient broth and the OD of biomass was observed at 660 nm against autoclaved media as blank. The OD of cultures was compared with control OD of culture (culture grown in the absence of acryflavin) and the concentration of acryflavin giving nearly 50% less OD was considered LD-50. The LD-50 value obtained in our results for *Escherichia coli* was 10 μg.

25 mL of nutrient broth was prepared, autoclaved, and inoculated with test organism and incubated at 370 °C for 18 h. Five tubes of nutrient broth containing LD-50 concentrations of acryflavin were prepared and inoculated with 18 h old test organism (1%) in tube no.1. The control tubes without acryflavin were also prepared and inoculated, as mentioned earlier. The tubes were incubated at 370 °C for 24 h. From tube no.1, 1% culture was inoculated to tube no.2 and incubated further for 24 h. This serial inoculation was continued for five generations. At every generation, the plasmid was isolated and run on 1% agarose gel to observe for the presence/absence of plasmid and simultaneously the sample was analyzed by HPLC for methomyl degradation.

2.5 Statistical Analysis

Statistical significance between the control and experimental data were subjected to analysis of variance (ANOVA) together with Dunnett's test ($P < 0.05$).

3 Results and Discussion

In the present study, the organism was inoculated in synthetic medium containing methomyl (10^{-3}–10^{-7} M) for 24, 48, 72, and 96 h and subjected to HPLC analysis where methomyl

Table 1
Biodegradation of methomyl by *Escherichia coli*

Group	Treatment concentration (M)	Degradation (%)				
		Duration (h)				
		(Control	24 (h)	48 (h)	72(h)	96(h)
I	10^{-7}	53.66 ± 0.02	7.67 ± 0.03*	6.05 ± 0.02*	2.67 ± 0.03*	0.91 ± 0.02*
II	10^{-6}	61.39 ± 0.05	8.26 ± 0.03*	7.11 ± 0.03*	4.84 ± 0.03*	2.38 ± 0.03*
III	10^{-5}	91.74 ± 0.03	18.67 ± 0.04*	17.13 ± 0.05*	16.49 ± 0.01*	14.83 ± 0.02*
IV	10^{-4}	96.04 ± 0.02	43.65 ± 0.02*	42.20 ± 0.02*	41.71 ± 0.02*	40.49 ± 0.02*
V	10^{-3}	98.55 ± 0.03	63.56 ± 0.03*	62.56 ± 0.04*	61.50 ± 0.02*	60.37 ± 0.05*

Values are mean ± SEM of 10 samples
*Significant $P \leq 0.05$ compared to control

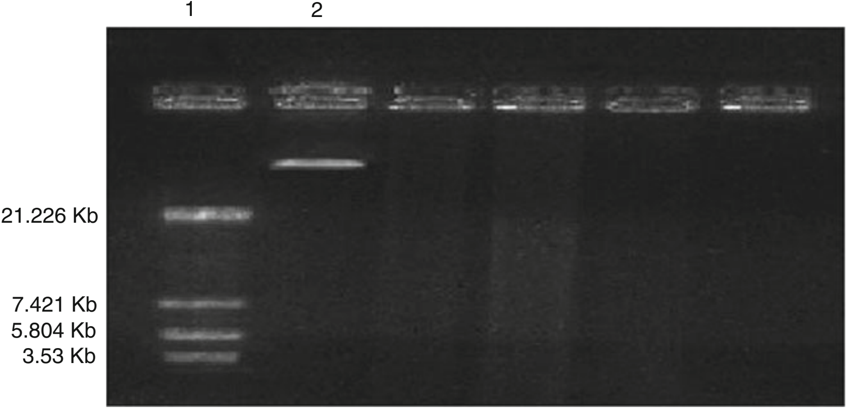

Fig. 1 Gel electrophoresis showing presence of plasmid in *Escherichia coli*

degradation (%) was observed. The present study on HPLC analysis at regular intervals of 24 h of *Escherichia coli* in the synthetic medium containing methomyl (10^{-3}–10^{-7} M) incubated for 96 h revealed that there was a significant decrease in the methomyl content in all the treated groups when compared with that of corresponding controls (Table 1). The plasmid isolated and cured cells of *Escherichia coli* were subjected to HPLC analysis at a regular interval of 24 h and simultaneously compared to the normal cells for five generations and the results were recorded. The present study reveals that the plasmid was isolated from *Escherichia coli* (Fig. 1) and subjected to curing. The plasmid was cured in the third generation (Fig. 2). The plasmid cured culture of *Escherichia coli* still acquired the ability to further degrade methomyl. Observing the HPLC results (Table 2), it can be deduced that the gene

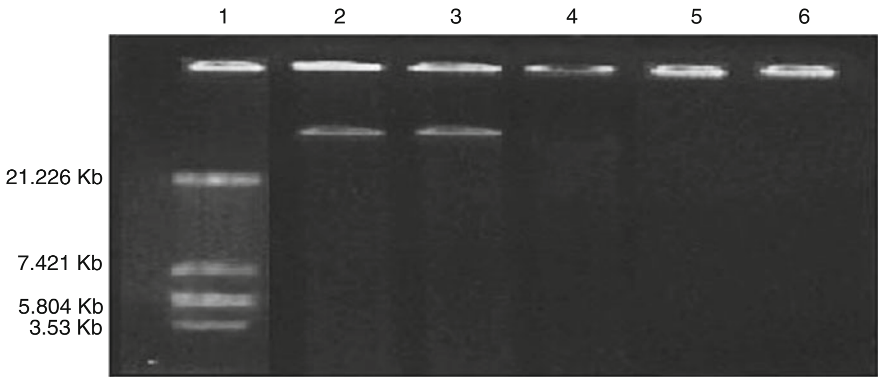

Fig. 2 Gel electrophoresis showing plasmid curing in *Escherichia coli*

Table 2
Plasmid curing of *Escherichia coli*

	Methomyl (%)	
Duration (h)	**Normal cells**	**Plasmid cured cells**
Control	28.65 ± 0.03	28.65 ± 0.03
24	24.36 ± 0.02*	26.62 ± 0.01*
48	23.25 ± 0.04*	25.16 ± 0.02*
72	18.17 ± 0.01*	24.89 ± 0.01*
96	14.59 ± 0.03*	23.91 ± 0.02*
120	12.15 ± 0.03*	22.39 ± 0.07*

Values are mean ± SEM of 10 samples
*Significant $P \leq 0.05$ compared to control

responsible for bioremediation process was not exclusively encoded in the plasmid alone and that multiple genes present both on the plasmid and the main genome of *Escherichia coli* may be involved in the bioremediation of methomyl. The discovery of microorganisms capable of tolerating or growing in high concentrations of pesticides provides a potentially interesting avenue for treating hazardous wastes [3, 19]. Investigations resulting in the identification of microbial isolates which are apparently responsible for the accelerated degradation of individual pesticides are necessary [9].

The present study revealed that the methomyl was decreased significantly by *Escherichia coli*. However, degradation rates differed. Further, it was observed that *Escherichia coli* exhibited a good growth rate and higher biodegradation efficiency toward methomyl, demonstrating its potential use in bioremediation. It is suggested that the detoxification metabolism occurs when a microorganism uses the pesticide as a carbon and energy source and the

process is facilitated by resistant microorganisms [19]. Earlier, two microorganisms classified as *Pseudomonas* sp. and *Escherichia coli* have been isolated and described as possessing the ability to degrade lindane. The results obtained in this study were in agreement with earlier reports that indicated the involvement of different species of *Enterobacteriaecae* in the degradation of insecticides like chlorpyrifos, phosphonate, and glyphosate [30]. It has been reported that the paraoxonbiodegradation by *Escherichia coli* strains was higher than those of non-*Escherichia coli* strains [31]. Das et al. [32] have studied the influence and persistence of phorate and carbofuran on *Escherichia coli* and reported its dissipation. It has been reported that *Stenotrophomonas maltophilia* M1 strain is capable of methomyl degradation [7]. Singh et al. [33] reported that *Enterobacter* strain B-14 used chlorpyrifos as a source of carbon and phosphorous. In the earlier studies, the capability of dimethoate degradation by an isolate of *Proteus vulgaris* was reported by Mandal et al. [3]. Utilization of chlorpyrifos by several soil bacteria has been reported [33], it was indicated that *P. stuartii* is capable of utilizing the organophosphorous pesticide chlorpyrifos as a source of carbon [30]. Bhagobaty et al. [34] have reported of the *Pseudomonas* strains capable of degrading chlorpyrifos. Bioremediation of endosulfan was carried out by *Staphylococcus sp*and *Bacillus circulans* [1]. Murugesan et al. [13] reported that a marked negative effect on the rate of degradation was observed with increased concentration of cypermethrin which may be due to mineral nutrients that are required for the growth of *Pseudomonas* and biodegradation of cypermethrin may become rate limiting in the wastewater sample. Their findings suggest that the utilization of cypermethrin by *Pseudomonas aerugi*nosa may be feasible and this treatment option for the removal of pesticide from the soil and degradation observed only in the presence of microorganisms.

The present bioremediation study using *Escherichia coli* cells showed higher percent degradation of methomyl, indicating that *Escherichia coli* is more efficient in methomyl degradation. Similarly, it was reported that *Escherichia coli* were used to degrade the organochlorine insecticide acetofenate and the organophosphorous insecticide chlorpyrifos and reported that *Escherichia coli* could degrade insecticides with ester bonds and it displayed different degradation rates with different pesticides [35]. Biodetoxifiction of coumaphos insecticide using *Escherichia coli* was reported by Mansee et al. [31]. It has been reported that individual reactions of degradation–detoxification pathways include oxidation, reduction, hydrolysis, and conjugation. Metabolic pathway diversity depends on the chemical structure of the xenobiotic compound, the organism, environmental conditions, metabolic factors, and the regulating expression of these biochemical pathways [36, 37]. The response of microorganisms to pesticides in pure culture was reported to be variable with no consistent relationship evident

between the source of the microorganisms and the observed response. Such a different pattern of pesticide utilization might be due to variation in the ecological niches or in the biochemical nature of pesticide degradation [38]. The observed changes in the activity depend on the intensity and spectrum of activity as well as persistence of the parent chemicals or its metabolites. Pesticides might affect microorganisms by reducing their numbers, biochemical activity, diversity and changing the microbial community structure [39].

The different degradation rates observed in the present study may be due to the chemical structure of the xenobiotic compound, the organism involved, environmental conditions, metabolic factors, and/or the regulating expression of these biochemical pathways [36, 37], no consistent relationship between the source of the microorganisms, toxicant and the observed response, variation in the ecological niches or in the biochemical nature of pesticide degradation [3] or diversity and changes in the microbial community structure [39, 40].

The results of the present study showed that with the plasmid curing the degradation of methomyl by *Escherichia coli* was not stopped indicating that the gene for methomyl degradation was not wholly plasmid coded and that multiple genes of both plasmid and main genome may be involved in the bioremediation. Similar results obtained by Park et al. [41] also indicated that the titanium resistance of *Escherichia coli* was not dependent on its plasmid DNA, while that of *Pseudomonas aeruginosa* was in fact dependent on its plasmid. It is suggested that the role of transposon(s) is important in flip flop of the genes (including chlorpyrifos degrading genes) and further reported that the biodegradation of chlorpyrifos is mediated by split location of the genes (located on the plasmid and the chromosome) in a soil isolate *Pseudomonas putida MAS*-1 [35]. Chromosome and plasmid encoded resistance genetic systems have been studied in *Pseudomonas,* as well as in related bacteria [42]. The responsible genes that degrade the organophosphate pesticides, such as methyl parathion, have been reported in the plasmid and in the chromosome of different bacteria such as *Pseudomonas, Pleisemonas, Bacillus,* and *Flavobacterium* [43]. A number of choices for co-expression of multiple target proteins in *Escherichia coli* are currently available, of which the plasmid duet has been designated for co-expression of target genes [44]. It has been reported that the genes encode the degradation of both naturally occurring and xenobiotic organic compounds are often located on plasmids, transposons, or other mobile and/or integrative elements [45]. The genetic information encoding these metabolic activities is often found on plasmids and/or other mobile elements [46].

Under natural conditions a number of routes exist for the widespread dissemination of genetic material through the bacterial

populations. With different resistant genes, this spread has been associated facilitated by transposable genetic elements and broad-natural host range plasmids. It has been reported that both on plasmids and in the chromosome, catabolic genes are often bordered by IS-elements (insertion sequences). These IS elements may have played a role in recruitment of these genes by the replicon but also increase the potential of further exchange of the genes between different replicons and different hosts. It has been already reported by Lan et al. [46] that the co-expression in *Escherichia coli* facilitates the identification and characterization of the interaction of different proteins preceded by different genes. The ready transfer of the degrading plasmids within the organism plays a major role in the dissemination of genetic information for the degradation and recycling of a number of complex compounds. The reason for this difference is not known. However, certain plasmids have shown to undergo transition to higher molecular weight species under certain growth conditions and another possible suggestion was that the plasmid may only encode the enzyme controlling one step in the degradative pathway and the remainder of the degradative pathway would be chromosomally located [35].

In the present study, the multiple genes present both on plasmid and main genome involved in methomyl degradation by *Escherichia coli* may be due to the presence of transposable genetic elements and broad-natural host range plasmids [47], split location of the genes, located both on the plasmid and the chromosome [35, 48], genes that are often located on plasmids, transposons, or other mobile and/or integrative elements [48] presence of insertion sequences, plasmids that undergo transition to higher molecular weight species under certain growth conditions [9] or due to the fact that the genetic information encoding these metabolic activities is often found on plasmids and/or other mobile elements [43, 49]. The present results confirm that the genes of the plasmid and the chromosome of *Escherichia coli* are involved in methomyl degradation which can be used as cloning vehicles in recombinant DNA technology and exploited for bioremediation.

4 Conclusion

This study demonstrated that the methomyl was degraded efficiently by *Escherichia coli*. This strain contains a plasmid and is believed to be partially responsible along with the main chromosome for the degradation of methomyl carrying the degrading gene. *Escherichia coli* can be exploited for bioremediation owing to its ease of culture and potentiality toward methomyl biodegradation or breaking down the pesticide molecules into small molecule fractions that may possibly be less harmful to the environment as well as more easy to be degraded biologically further having selective advantage under a given environmental state.

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Chapter 5

Burkholderia Sp. Strain BBK_9: A Potent Agent for Propiconazole Degradation

Praveen P. Satapute and Basappa B. Kaliwal

Abstract

Propiconazole, a leading triazoles fungicide, used to protect the standing plants from fungal diseases. The residues of this fungicide are known to persist in agricultural soil for longer duration. Therefore, in the present study, a newly isolated potent bacterium *Burkholderia* sp. strain BBK_9 was opted for the degradation of propiconazole (20 μg/mL) by immobilization process. The effect of propiconazole (10, 20, 30 μg/mL) on nucleic acids (DNA and RNA), glucose, and stress enzymes of BBK_9 strain was investigated. Furthermore, the induction of different proteins during the degradation of propiconazole was elucidated by the SDS PAGE analysis. Molecular functional dissimilarity in BBK_9 strain when exposed propiconazole was examined by the Fourier Transmission Infrared Spectroscopy (FTIR). The results indicate that immobilized cells of BBK_9 strain utilized propiconazole up to 19.2 μg/mL after 96 h at 30 °C and pH 7. Moreover, the highest concentration of propiconazole (30 μg/mL) was effected BBK_9 strain, and contents of DNA, RNA, and glucose were significantly decreased, additionally, stress enzymes were significantly increased at 30 μg/mL of propiconazole. The bacterial cells were aided by the secretion major proteins between 43 and 29 kDa in the degradation process. Production of molecular functional groups was founded more in the propiconazole treated strain than in the untreated bacterial strain. These outcomes revealed that *Burkholderia* sp. BBK_9 strain was found to be promising agent for remediation of pollutants; meanwhile, the excess of concentration will also be harmful for the soil health and microbial community.

Key words Propiconazole, Biodegradation, Immobilization, Nucleic acids, SDS PAGE, FTIR

1 Introduction

Indian agriculture history dates back to the Indus valley civilization era. Presently, India is the second ranked nation in output of farm products. Agriculture sector alone contributes 10–15% of Indian GDP (Gross domestic products) [1]. An implementation of newer technologies like bio fertilizer and pesticides in farm made the Indian agriculture sector reach its glorious heights in the world [2]. Further, the remediation of persistent pesticides from soil is found to be a major agricultural problem [3], to overcome this problem many technologies were introduced, although scenario

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seems to be persistent for longer duration. Interestingly, the mode of pesticide effects in the present days is reduced because of execution of microorganisms in the field of agriculture [4]. Moreover, in recent years, microorganisms were frequently used as a biological weapon for the removal of contaminants that were present in the soil [5–7]. However, some potential microbes may fail to elucidate the pollutants from the sites, because of the presence of the heavy amount of pollutants and this state of anxiety would make microbes eliminate from the sites or microbes may undergo many stress conditions, the molecular functional dissimilarity is also a possible threat that can attack microorganisms under the stress condition.

Propiconazole, a triazole group of fungicide, is famously known as TILT 25EC. It is a systemic fungicide with a broad range of activity. Formally, it is sprayed on the mushroom, rice, oats, peaches, corn, peanut, etc. on cereals, propiconazole controls diseases caused by *Pyrenophora teres, Rhynchosporium secalis, Erysiphe graminis*, *Septoria* spp., *Leptosphaeria nodorum*, *Puccinia* spp., and *Pseudocerosporella herpotrichoides* [8, 9]. Propiconazole is recommended as a foliar spray, further, the residues of this fungicide will reach to soil by drifting from the plant parts. Additionally, soil will grasp the residue of propiconazole during the application [10] and it will be persistent in soil for longer duration (96 to 575 days). To overcome this problem, the present study was undertaken to degrade the propiconazole by immobilized cells of *Burkholderia* sp. BBK_9 strain, propiconazole effect on bacterial biochemical content were investigated. Additionally, stress enzymes, expression of proteins during stress conditions, and molecular dissimilarity under stress were studied.

2 Materials and Methods

2.1 Chemicals and Reagents

Propiconazole (technical grade) fungicide with the purity of 94% was procured from Nagarjuna Agrichem PVT. Ltd. (Srikakullam, India). Acetonitrile and Ethyl acetate used were of maximum analytical and HPLC grades. The Seubert's mineral salts medium (MSM) [11] for the degradation study and Nutrient broth (Himedia) for the cultivation of bacterium was used.

2.2 Microorganism

Burkholderia sp. strain BBK_9 previously identified in our laboratory by the microbial enrichment culture method was selected for the present investigation [12]. The bacterium was stored at 4 °C on the slants of Seubert's mineral salts agar medium.

2.3 Medium

For the investigation of propiconazole biodegradation by the immobilization, a loop full of fungicide resistant bacterial culture (BBK_9 strain) was inoculated into the 500 mL Erlenmeyer flask

containing 100 mL of sterile nutrient broth. During the exponential growth phase, 1 mL of aliquot was aseptically inoculated to the 100 mL of sterile MSM (pH 7) containing 20 μg/mL of propiconazole concentration as sole carbon source and incubate at 30 °C on the rotary shaker at 140 rpm. Meanwhile, growth of the BBK_9 strain was regularly monitored.

2.4 Immobilization of Cells Burkholderia Sp. Strain BBK_9

2.4.1 Cells Harvesting

Cells of propiconazole utilizing strain BBK_9 were harvested during the exponential phase, i.e., 1 mL of bacterial liquid culture containing 3×10^6 cfu/mL of live cells. The bacterial suspension was centrifuged at 10,000 × *g* for 10 min at 4 °C, the supernatant was discarded, and the cells were repeatedly washed with phosphate buffer containing the strength of 50 mM with pH 7. Further, the cells were immobilized by the sodium alginate beads.

2.4.2 Sodium Alginate (SA) Entrapment of Cells

For the SA entrapment of cells, the Bettman and Rehm (1984) [13] method was adopted. SA (4% w/v) was liquefied in water at 100 °C after the complete mix, the SA was autoclaved for 15 min at 121 °C. A bacterial cell suspension of 25 mL was aseptically inoculated to 500 mL Erlenmeyer flask containing 100 mL of sterile MSM and mixed by vigorous stirring. The combination of SA and bacterial cells was added to cold 0.2 M $CaCl_2$ solution dropwise through a glass burette for the extrusion of SA. Approximately diameter of 3 mm gel beads was obtained. Further, obtained gel beads were again added to fresh 0.2 M $CaCl_2$ solution for 8 h with mild agitation and the gel beads were stored at −20 °C for 24 h. Lastly, the frozen gel beads were washed several times with distilled water and stored at 4 °C until the further use.

2.4.3 Biodegradation Studies

SA gel beads of 12 g were added to an Erlenmeyer flask containing 100 mL of sterile autoclaved MSM with 20 μg/mL of propiconazole a sole carbon source and the same concentration of propiconazole without SA beads were served as control.

2.4.4 Preparation of Analytical Solutions

At the regular interval of time, from the spent medium 5 mL of culture was taken out and subjected to centrifugation at 10,000 × *g* for 15 min at 4 °C and propiconazole/metabolites were extracted according to the method described in our previously published literature [12]. The ethyl acetate extraction was implemented to evaluate the spent concentration of propiconazole by the immobilized cells of BBK_9 strain, the residues dissolved in the acetonitrile were subjected to the HPLC analysis.

2.4.5 Analytical Conditions

The residues of propiconazole were dissolved in acetonitrile, the degradation rate was observed in UV spectrophotometer at 220 nm. Further, the rate of consumed propiconazole was confirmed by LC-UV analysis. Further, to ratify the degradation of propiconazole, an aliquot of 5 μL of standard propiconazole and

24 μL samples was injected. Agilent Compact 1120 containing C 18 column with variable wavelength UV indicator and aliquots were eluted at 1.2 min/mL with the combination of water and acetonitrile (20:80).

2.5 Effect of Propiconazole on the Biochemical Content of *Burkholderia* Sp. Strain BBK_9

2.5.1 Sample Preparation

Burkholderia sp. strain BBK_9 was grown in the MSM amended with various concentrations of propiconazole (10, 20, 30 μg/mL) and incubated at 30 °C at 140 rpm, at regular interval of times 5 mL of culture was withdrawn and centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatant was used for further studies.

2.5.2 Effect of Propiconazole on the Nucleic Acid, Protein, and Glucose

DNA Estimation

Estimation of DNA was performed according to the method of Grossman and Moldave (1968) [14]. Briefly, 2 mL of culture filtrates was mixed with 4 mL of the diphenylamine reagent with gentle shaking followed by the vortex. The mixture was incubated in the boiling water bath for 10 min and allowed to cool at room temperature, the absorbance of blue color developed was recorded against reagent blank at 595 nm in a photometer.

RNA Estimation

Estimation of RNA was performed according to the method of Grossman and Moldave (1968) [14]. To 3 mL of the culture filtrate 3 mL of orcinol reagent was added with constant shaking followed by the vortex. Tubes were incubated at boiling water bath for 30 min and allow the tube to cool at room temperature and green color developed was recorded against blank at 665 nm in a photometer.

Protein Estimation

Protein was estimated by the method of Lowry et al. (1951) [15]. Briefly, to 1 mL of the culture filtrate, 5 mL of alkaline copper reagent was supplemented with gentle mixing, further the mixture was allowed to stand at room temperature for 20 min, 0.5 mL of Folin's reagent (FCR) was added and mixed by the gentle shaking, tubes were incubated at room temperature for 30 min. The color formation was recorded by the photometer at 660 nm against the water blank.

Estimation of Glucose Utilization

Utilization of glucose by the *Burkholderia* sp. BBK_9 strain was estimated by Anthrone method which was previously described by Scott and Melvin (1953) [16]. From 24 hold liquid culture medium, 0.1 mL of sample was withdrawn aseptically and diluted up to 1 mL with sterile distilled water and 4 mL of anthrone reagent was added, the tube was mixed thoroughly and incubated over a boiling water bath for 5 min and allow the tubes to cool at room

temperature, a dark green colored product was formed and its optical density was recorded at 620 NM against water blank.

2.6 Effect of Propiconazole on the Stress Enzymes of Burkholderia Sp. BBK_9 Strain

2.6.1 Extraction of Enzymes

For the extraction of cell-free filtrates the method described by Talwar et al. (2013) [17] was followed. *Burkholderia* sp. BBK_9 strain cells were washed and grown in Tris–HCl buffer pH 6.8 amended with 1 μg/mL propiconazole and sonicated (Sonics vibra cell) for 5 min and centrifuged. The supernatant was used for the enzyme assay.

2.6.2 Superoxide Dismutase (SOD)

The activity of SOD was estimated by the method of Beauchamp and Fridovich (1971) [18]. Culture extracts of different ranges, i.e., 0–150 μL were added to tubes containing 50 Mm phosphate buffer pH 7.8, 13 mM methionine, 75 mM nitrobluetetrazolium chloride monohydrate (NBT), 0.1 mM EDTA, and 2 μM riboflavin. Reaction mixtures were thoroughly mixed and tubes were incubated near light. The activity of SOD was measured at 560 nm. Based on the inhibition of NBT by volume of enzyme extracts, one unit of the enzyme was calculated and it was expressed as μmoles per mL per min.

2.6.3 Catalase (CAT)

Catalase activity of BBK_9 strains was examined using the method employed by the Sadasivam and Manickam (1996) [19]. Culture filtrates of volume 25 μL were added to the tubes containing 50 Mm phosphate buffer pH 7.0 and 15 mM hydrogen peroxide. The activity of catalase was measured at 240 nm against the control at regular interval of every 30 sec up to 3 min. The activity catalase was calculated using the following formula:

Activity $= (\text{O.D} \times 40\ \mu\text{moles/mL/min})/0.36 \times 1 \times \Delta t \times$ vol. of reaction mixture.

2.7 Protein Expression During the Degradation Propiconazole

2.7.1 SDS-PAGE analysis

Expression of protein during the biotransformation of propiconazole in MSM by BBK_9 strain was analyzed by the SDS-PAGE by employing the method of Laemmli (1970) [20]. At the regular intervals 5 mL of culture was withdrawn and prepared sample for SDS- PAGE analysis, adopting the method of Talwar et al. (2013) [17]. Stalking and separating gel of 10% were prepared with the distilled, Acrylamide (30%), SDS, APS, TEMED and Tris–Cl Buffer (1.5 M). The mixture of stacking gel was dispensed onto resolving gel and comb was inserted; further, it was carefully detached from the polymerized gel. The cast gel was placed in Poly Acrylamide Gel Electrophoresis Unit and run buffer (Tris- 3 g, Glysine- 14.3 g, SDS- 2.0 g for 1 L) was poured. The samples were treated with sample buffer (1.5 M Tris–Cl (pH 8.0) 0.625 mL, 20% SDS 1.0 mL, Glycerol 1.0 mL, 2-mercaptoethanol, 0.5 mL, 0.2%

Bromophenol blue) in 1:1 ratio and heated in boiling water bath for 10 min. The sample was carefully loaded onto the gel and ran at constant voltage of 100 V for 3 h. Further, the gel was carefully removed and placed in Staining solution, i.e., Methanol 4 mL, distilled water 5 mL, glacial acetic acid 1 mL, 0.2% Coomassie Brilliant Blue on the rocker for 12 h. Further, the protein expression was observed in the gel documentation.

2.8 Molecular Dissimilarity of BBK_9 Strain When Treated with Propiconazole

2.8.1 FT-IR Analysis

Prior to implying, the microbial molecular dissimilarity under stress conditions, 100 mL of 24 h old bacterial culture was centrifuged at 10,000 × *g* for 15 min, the supernatant was discarded and biomass was used for the lyophilization using liquid nitrogen. Around 4 mg of frozen bacterial biomass was finely powdered and mixed with dry KBr, the formed extrusion was subjected to Nicolet FT-IR 6700 (Thermo scientific). The spectra's were obtained in a turbo mode with the DTGS-KBr sensor in the spectrometer range from 4000 Cm^{-1} to 400 Cm^{-1} of ETC source. Resultant spectra's were compared with the standard QC OMNIC libraries [21].

3 Results and Discussion

3.1 Biodegradation Propiconazole by Immobilized Cells of Burkholderia Sp. BBK_9 Strain

The bacteria strain BBK_9 was investigated for its degradation capacity. Information shows that immobilized cells were degraded propiconazole of up to 19.2 μg/mL after the incubation of 96 h in 30 °C at pH 7 when compared to the uninoculated controls (Fig. 1). The standard propiconazole of 50 μL/mL was injected to HPLC and the single was eluted at 3.41 min of retention time. Further, culture extracts withdrawn at different interval show different peak patterns and the standard propiconazole peak was decreased in the area count when compared to the other peaks (Fig. 2).

3.2 Effect of Propiconazole on the Nucleic Acids of Burkholderia Sp. Strain BBK_9

3.2.1 DNA Estimation

The effect of propiconazole on the DNA concentration of *Burkholderia* sp. BBK_9 strain is given in Fig. 3. The DNA content in the control (without exposure to propiconazole) was found to be 102.13, 147.53, and 169.08 μg/mL after the desired incubation period of 24, 48, and 72 h respectively. The DNA concentration of bacterial cells treated with 10 μg/L was found to be 85.73, 128.63, and 141.18 after the incubation period of 24, 48, and 72 h respectively. Similarly, the DNA concentration in the bacterial cells treated with 20 μg/mL was 59.03, 91.68, and 88.63 μg/mL after 24, 48, and 72 h of giving incubation respectively. The bacterial cells treated with 30 μg/mL exhibit the low quantity of DNA content when above-mentioned concentrations of propiconazole (10 and 20 μg/mL) and the concentration of DNA were found to be 27.63, 64.12, and 57.96 μg/mL after 24, 48, and 72 h of incubation respectively.

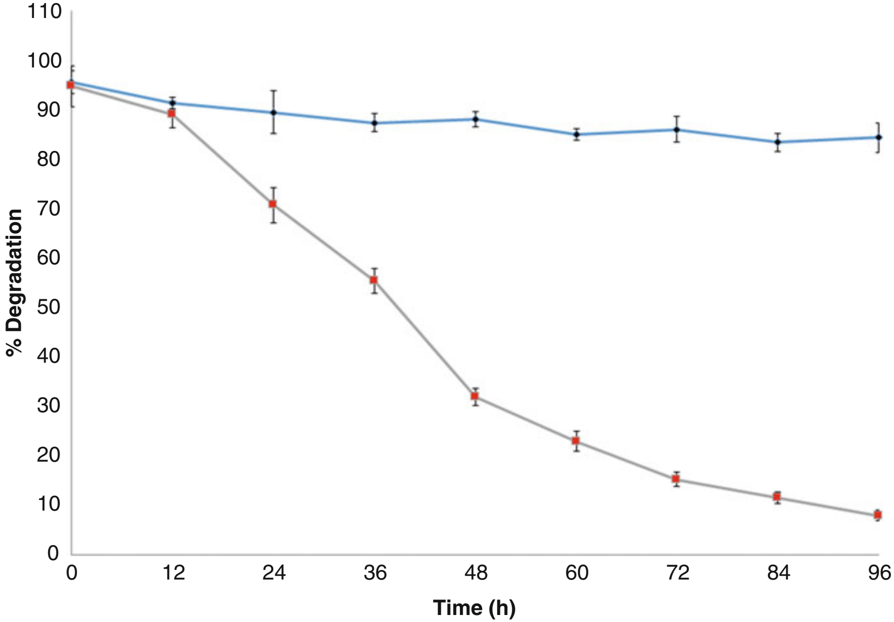

Fig. 1 Biodegradation of propiconazole by immobilized cells of BBK_9 strain. Percentage of propiconazole degradation (–■–) VS uninoculated control (–●–). *Error bars* represents mean ± standard error (SE) of three independent replicates

3.2.2 RNA Estimation

The effect of propiconazole on the RNA concentration of *Burkholderia* sp. BBK_9 strain is given in Fig. 4. In the control (without treating with propiconazole) the RNA concentration of *Burkholderia* sp. BBK_9 strain was found to be 24.59, 53.50, and 76.64 μg/mL in desired incubation period of 24, 48, and 72 h respectively. Meanwhile, the cells BBK-9 strain treated with 10 μg/mL of propiconazole was exhibiting the increasing pattern of RNA contents with increases in duration and content of RNA was found to be 25.83, 36.42, and 41.74 μg/mL at 24, 48, and 72 h respectively. The bacterial strain exposed to 20 μg/mL of propiconazole showed a different quantity of RNA at different durations and the concentration of RNA was found to be 19.58, 27.72, and 32.91 μg/mL after the given incubation period. Propiconazole of 30 μg/mL strength was proven a toxic force on the RNA content and it was found to be 11.2, 15.25, and 17.18 μg/mL after 24, 48, and 72 h respectively.

The higher doses of propiconazole were found to be toxic to nucleic acid production during the metabolisms of BBK_9 strain. It was observed that Burkholderia sp. BBK_9 strain was adopted itself for the lower concentration (10 μg/mL) of propiconazole and activity slightly affected. Previous report on the chlorpyrifos and cypermethrin pesticides suggested that exposure of constant and higher pesticide residues to the microbe will have adversely impacted the DNA and RNA contents [22]. In the same way, butachlor, a

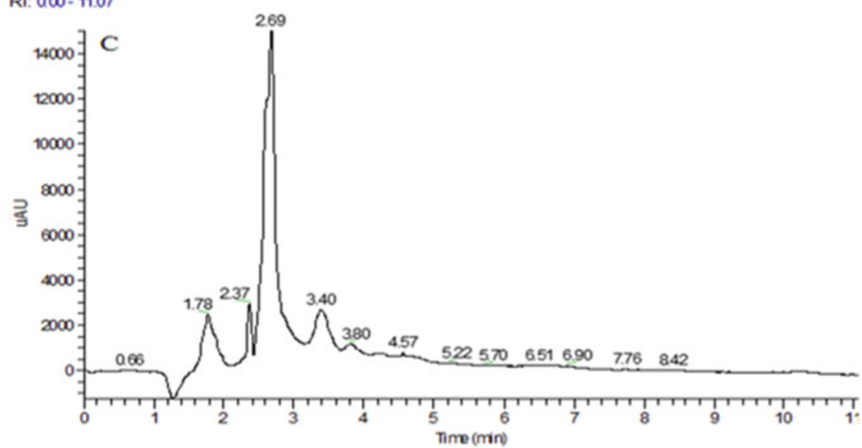

Fig. 2 HPLC profile of propiconazole during degradation by the immobilized cells of *Burkholderia* sp. BBK_9 strain. Propiconazole Extracted at 0 h (**a**), propiconazole residual concentration analyzed at 48 h (**b**) and 19.2 μg/mL of propiconazole degradation at 96 h (**c**)

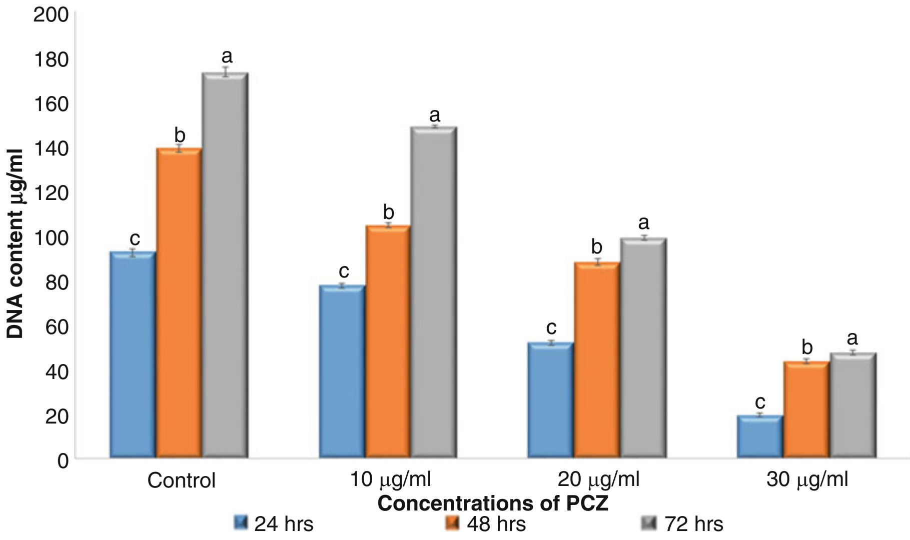

Fig. 3 Effect of propiconazole treatment on DNA content in *Burkholderia* sp. BBK_9. Data are the means ± S.E of three independent replicate for each incubation period. Means followed by the different letter are significantly different from each other according to Tukey's test ($P < 0.05$)

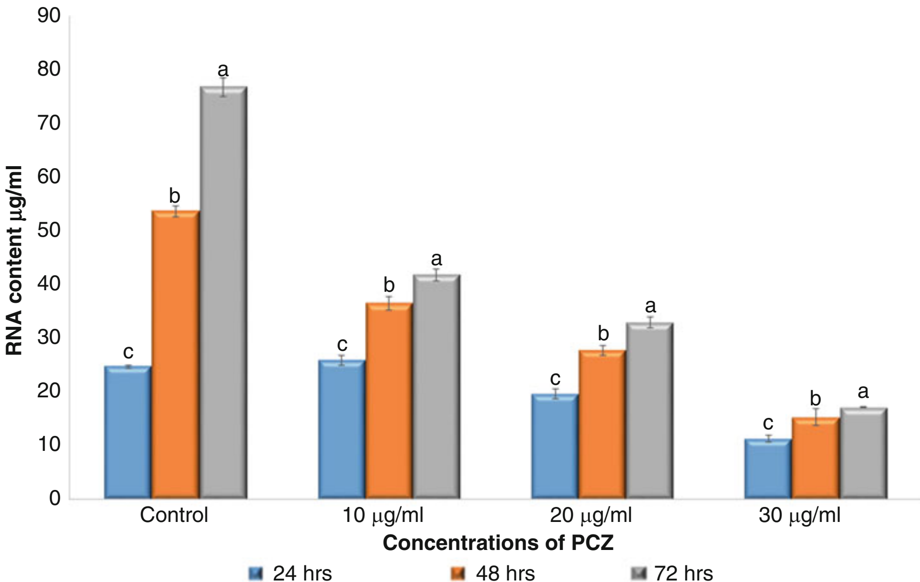

Fig. 4 Effect of propiconazole on the RNA content in *Burkholderia* sp. BBK_9 strain. Data are the means ± S.E of three independent replicates for each incubation period. Means followed by the different letter are significantly different from each other according to Tukey's test ($P < 0.05$)

toxic pesticide, belongs to the group of acetanilide herbicides. This herbicide is an able toxicant that has the ability to affect the biochemical parameters of *Azospirillum* sp. Contradictorily, a very few chemicals have an ability to enhance the activity of microbes.

Table 1
Effect of propiconazole on the protein content in *Burkholderia* sp. BBK_9 strain

		Protein Content (mg/mL)		
		Duration (h)		
Group	Treatment concentration (μg/L)	24	48	72
I	Control	1.40 ± 0.03*	1.54 ± 0.02*	1.27 ± 0.01*
II	10	1.31 ± 0.08	1.26 ± 0.07*	1.13 ± 0.03*
III	20	1.33 ± 0.05*	1.24 ± 0.01*	1.39 ± 0.20
IV	30	1.2 ± 0.03*	1.18 ± 0.06	1.10 ± 05*

Values are mean ± S.E.M ($n = 3$)
*Significant $P \leq 0.05$ compared to control

Similarly, carbofuran was found to an effect agent for the enhancement of bacterial metabolism [23]. It was understood that our experimental results that fall under the category of toxic pesticides will have the ability to reduce the growth and biochemical parameters significantly.

3.2.3 Protein Estimation

The propiconazole effect on protein content of *Burkholderia* sp. strain BBK_9 is given in Table 1. In the group of controls protein concentration was found to be 1.40, 1.54, and 1.27 mg/mL at desired incubation period. Conversely, the medium amended with 10 μg/mL showed protein concentrations 1.31, 1.26, and 1.13 mg/mL at 24, 48, and 72 h respectively. The strain treated with 20 μg/mL of propiconazole showed a protein concentration of 1.33, 1.24, and 1.39 in a 24, 48, and 72 h of incubation period. Likewise, 30 μg/mL of propiconazole also favored the isolate to express the protein concentration like 1.28, 1.18, and 1.10 at 24, 48, and 72 h respectively.

It was noticed that, the higher residual concentration of propiconazole was significantly reduced the content of protein in BBK_9 strain. Similar studies on toxic compounds have reported that, protein concentrations during bacterial growth was suppressed with contact of toxicants [24]. Moreover, few pesticides like pirimicarb, isoproturon, captan and deltamethrin have the capability to reduce the protein content in bacteria [25]. It is was well known that, the endosulfan a toxic pesticide with many adverse impact of microbes have exhibited it ability to decrease the population of bacteria in soil and affects the metabolism of bacterial community [26]. The experimental data of our studies reports that, when compared with controls, treated groups showed low levels of protein content, hence it was understood that, the propiconazole was inhibited the protein content in the strain.

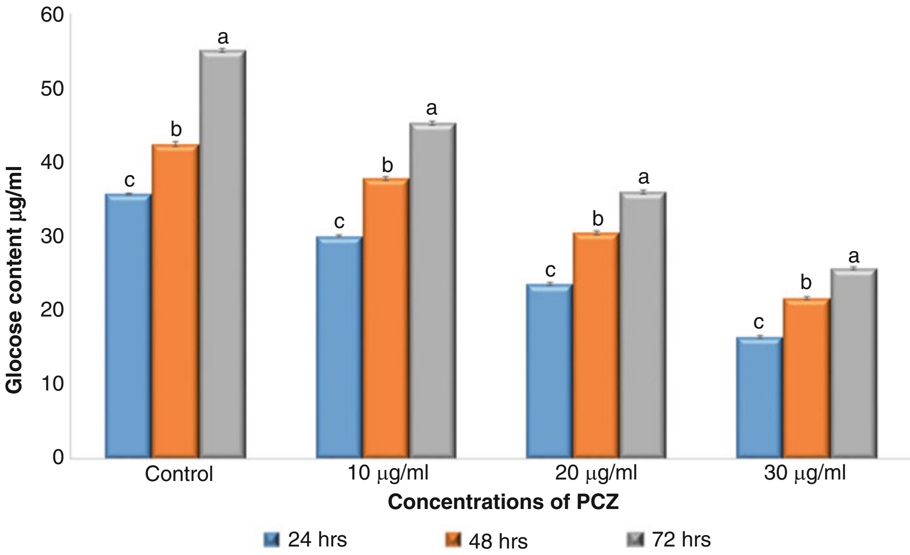

Fig. 5 Effect of propiconazole on utilization of glucose by *Burkholderia* sp. BBK_9. Data are the means ± S.E of three independent replicates for each incubation period. Means followed by the different letters are significantly different from each other according to Tukey's test ($P < 0.05$)

3.2.4 Estimation of Glucose Utilization

The utilization of glucose by the *Burkholderia* sp. BBK_9 strain is given in the Fig. 5. In the control (without treating with propiconazole) glucose utilization by *Burkholderia* sp. BBK_9 strain was found 35.65, 42.41 and 55.03 µg/mL in desired incubation period of 24, 48 and 72 h respectively. Meanwhile, the cells BBK-9 strain treated with 10 µg/mL of propiconazole was exhibiting the increasing pattern of Glucose utilization with increases duration and was content of glucose was found to be 29.98, 37.75 and 45.20 µg/mL at 24, 48 and 72 h respectively. The bacterial strain exposed to 20 µg/mL of propiconazole showed a different amount of glucose at different duration and the concentration of glucose was found to be 23.55, 30.41 and 35.94 µg/mL after the given incubation period. Propiconazole of 30 µg/mL strength was showing little toxic effect on the glucose utilization and it was found 16.52, 21.76 and 25.59 µg/mL after 24, 48 and 72 h respectively.

The decrease in the concentration of glucose in microorganism with increased concentrations and time of contact with propiconazole may due to the toxic impact of propiconazole on cells of *Burkholderia* sp. BBK_9 strains. Similarly, Cabiscol et al. (2000) [27] have reported the same kind of observation and it was said that toxic compounds have the ability to disturb the regular mechanism of microorganisms and will cause the detriment in the content of glucose and biochemical parameters. Interestingly, relative oxygen species (ROS) will also damage the utilization of glucose and other biochemical parameters, resulting in the cell death when the level of

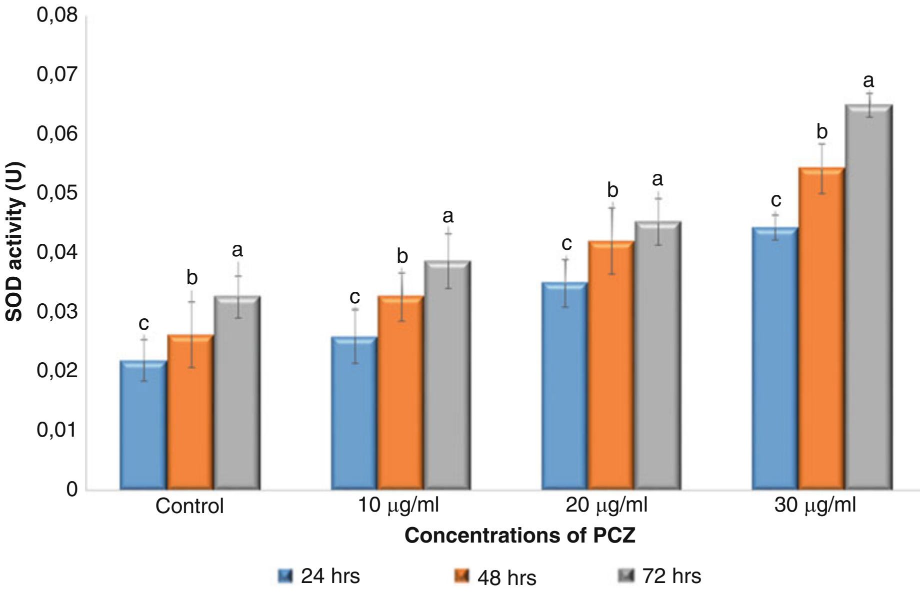

Fig. 6 Effect of propiconazole on SOD activity of *Burkholderia* sp.BBK_9 strain. Data are the means ±S.E of three independent replicates for each incubation period. Means followed by the different letters are significantly different from each other according to Tukey's test ($P < 0.05$)

ROS exceeds a microorganism detoxification [28]. The significant detriment in the glucose concentration in the present study may be due to glucose starvation and oxidative stress and disturbance in cellular metabolism and modification of carbohydrate [29]. Soil isolate *Burkholderia* sp. BBK_9 strain was significantly reduced the content of glucose with dose and durational exposure to propiconazole. In addition, it was observed that the Significativity of obtained results suggest that, the propiconazole have adverse impact over the production of glucose by the Burkholderia sp. BBK_9 strain.

3.3 Effect of Propiconazole on the Stress Enzymes of Burkholderia Sp. BBK_9 Strain

3.3.1 Superoxide Dismutase (SOD)

The activity of SOD in the *Burkholderia* sp. BBK_9 strain in the control group SOD activity was found to be 0.022, 0.026 and 0.032 U at time period of 24, 48 and 72 h respectively. On the contact with 10 µg/mL of propiconazole the activity of SOD was achieved to be 0.026, 0.032 and 0.038 U at the desired period of 24, 48 and 72 h respectively. On exposure with 20 µg/mL of propiconazole showed an activity of 0.035, 0.042 and 0.045 U at the chosen period of 24, 48 and 72 h respectively. On the treatment with 30 µg/mL of propiconazole the activity of SOD was found to be 0.044, 0.054 and 0.065 U in 24, 48 and 72 h of incubation period respectively (Fig. 6).

3.3.2 Catalase

The activity of CAT in the *Burkholderia* sp. BBK_9 strain in the control group CAT activity was found to be 14.64, 16.21 and 18.45 U at time period of 24, 48 and 72 h respectively. On the

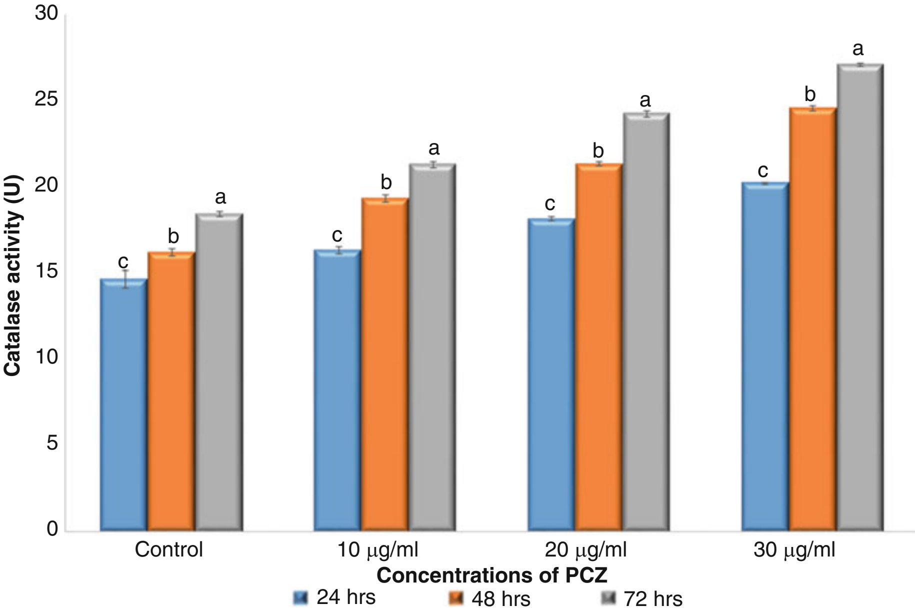

Fig. 7 Impact of propiconazole on catalase activity of *Burkholderia* sp. BBK_9 strain. Data are the means ±S.E of three independent replicates for each incubation period. Means followed by the different letters are significantly different from each other according to Tukey's test ($P < 0.05$)

contact with 10 µg/L of propiconazole the activity of CAT was achieved to be 16.34, 19.34 and 21.31 U at the desired period of 24, 48 and 72 h respectively. On exposure with 20 µg/L of propiconazole showed an activity of CAT 18.17, 21.36 and 24.25 U at the chosen period of 24, 48 and 72 h respectively. On the treatment with 30 µg/L of propiconazole the activity of CAT was found to be 20.23, 24.44 and 27.10 U in 24, 48 and 72 h of incubation period respectively (Fig. 7).

The levels of SOD in *Burkholderia* sp. slightly decreased. Conversely, oxidative stress response to the number pesticides and pollutant have been widely studied [30, 31]. Importantly, Park et al. (2006) [32] have carried out a research that affects the SOD concentration in the bacteria and it was suggested that the usage of higher dose will decrease the activity of SOD and other stress enzymes. In our study it was also shown that the higher concentration of propiconazole (30 µg/L) affected the concentration of SOD in *Burkholderia* sp. BBK_9 strains. In lower concentration of propiconazole (10 and 20 µg/L), the activity of SOD was significantly increased with increased duration (24, 48 and 72 h). However, in the soil isolate treated with 30 µg/mL of propiconazole showed the significantly lower activity of SOD. Thus, SOD activity was expressed significantly when it was treated with recommend doses. To date, the oxidative stress replies to numerous contaminants have been broadly inspected in bacteria [33]. CAT

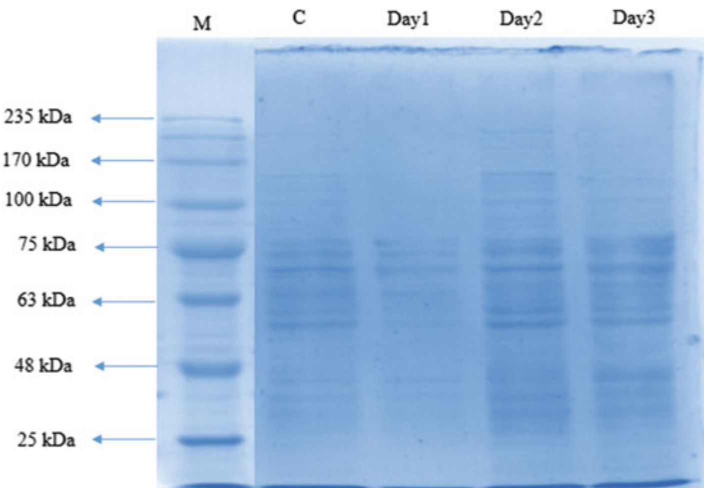

Fig. 8 Protein profiling of *Burkholderia* sp. BBK_9 strain during the course propiconazole degradation M-Marker, C- untreated, Day1-Protein extraction at first of propiconazole degradation, Day2-Protein extraction at second of propiconazole degradation, Day3-Protein extraction at third of propiconazole degradation

treated with lower concentrations of atrazine seemed to be unresponsive. While, Gram-negative bacterium *E. coli* K12 exhibited higher sensitivity to atrazine stress. Consequently, Gram-negative bacterium *E. coli* K12 is fit organism for studies about the act of atrazine stress. In contrast, in the lower concentration the activity of CAT was found promisingly good in our study, *Burkholderia* sp. BBK_9 strains share usual mode of action while the utilization of propiconazole as source of carbon. Hence, we believe that both organisms used in the present study may have good impact over the stress responses. In our study, it is possible that stimulation of CAT activity donates to the removal of ROS from the bacterial cell induced by propiconazole treatment. In the present study, the CAT activity was significantly enhanced in the treated groups of propiconazole (10, 20 and 30 μg/mL) at 24, 48 and 72 h and in controls the activity was significantly lowered.

3.4 Protein Expression During the Degradation Propiconazole

Protein expression during the path of degradation propiconazole was investigated and it was revealed that, the most of the protein expressed was falling between the ranges of roughly 170–40 kDa. It was also mentioned that, protein spots in the control lane were found similar to the propiconazole treated groups and it was also revealed the few spots were present in the range between 48 and 25 kDa (Fig. 8). The degradation of is characteristically valuable process, since the response of microbes that coverts pesticides in to less toxic and environmentally inactive. Additionally the involvement of pesticide resistance microbe and enzymes open up the new chances opportunity for spreading the extensive range of toxic pesticides that can be biodegraded in the upcoming days.

The earlier information on the protein studies suggest that, the *E. coli* exposed to the methomyl yielded the different protein when compared with control and also it was reported that, investigation of stress proteins is a favorable method for the examination of pesticides lethal intensities on the microbes [34]. On the other hand, the protein sources which were not exposed with methomyl were hardly expressed. However, in our study protein samples which were not treated with the propiconazole (control) were also expressed proteins. The earlier information detailed that, the toxic effect of certain toxicants will affect the polypeptide chains [35]. Additionally, the impact of various pesticides, will influences the outermost membrane protein expression, such as antibiotic treatment, mutation, changes in the environment, lipopolysaccharide modification and biofilm formation.

3.5 Functional Group Annotation of BBK_9 Strain

It was observed that *Burkholderia* sp. BBK_9 strain treated with 30 μg/mL propiconazole exhibits various molecular functional groups while the control without the exposure of propiconazole was not shown many responsive molecular functional groups. The main spectral annotation in control and treated groups was falls between the absorbance wave number regions at 3500–3000 cm^{-1} O–H stretching with bimolecular interpretation of enhanced cell was hydration, the major stretching of band between the absorbance of 2950–2700 cm^{-1} C–H stretch resulting in the formation of asymmetric fatty acids methyl, methylene groups, fatty acids methyl bonds and Saturated fatty acids methyl groups. The spectral range between 1780 and 1450 cm^{-1} containing C=O, C–N, C–O–P, C=C, C–H and COO molecular functional groups and the accumulation of amide groups indicate the formation of bacterial cell wall and fatty acids. Other minor functional groups like PO^{-2} band, C–O–P, CH–O band, C–O–P (str) esters bond and S–H, C–S band were elevated at the spectral region (Fig. 9). Our experimental results suggest that oxidative stress during the exposure of bacterial strain to toxicant will have adverse impact on the bacterial cell membrane and in cellular organization. The constant exposure of toxicants will lead to permanent consequences within the microbiota. In this specific circumstance and under certain metabolic activities, the microbes will adapt themselves to the toxic environment.

Acknowledgment

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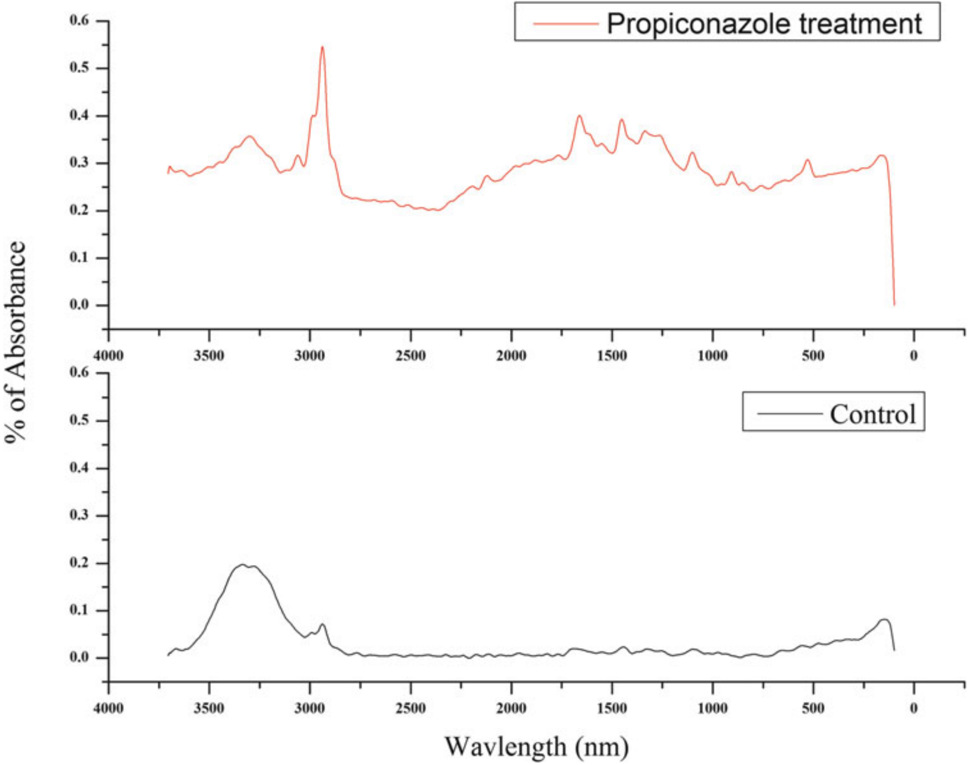

Fig. 9 FT- Raman spectra showing the effect of propiconazole on the cell wall of Burkholderia sp. BBK_9 strain

to the UGC-UPE fellowships and Post Graduate Department of Studies in Microbiology and Biotechnology, Karnatak University Dharwad for providing the laboratory facilities.

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Chapter 6

Application of Cell Immobilization in Slurry-Phase Bioremediation: Phenanthrene Biodegradation and Detoxification

Ali Partovinia and Fereshteh Naeimpoor

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are among the detrimental soil contaminants worldwide. Free microbial cells have been widely used for PAHs biodegradation in contaminated soils. However, only few studies have been carried out on the application of the immobilized cell system for bioremediation of PAH contaminated soil/slurry. In this chapter, we reviewed the literature on PAHs biodegradation, especially in solid/slurry phase by immobilized cell systems. This was followed by our experimental study on bioremediation of a clayey soil contaminated with phenantherene, a model contaminant, at 250, 500, 1000, and 2000 (mg PHE)(kg dry soil)$^{-1}$ using immobilized cell (IC) and free cell (FC). Formation of intermediate metabolites (IMs) of the PAH oxidation pathways was also examined since some of the IMs could be reluctant to further biodegradation or be more toxic than the parent pollutants. This was performed by measuring the total organic carbon (TOC) of soil and chemical oxygen demand (COD) of aqueous phase. Additionally, the effect of temperature (10, 20, 30, 40 °C), pH (5, 7, 9), and inoculum size (300 and 600 mg/L) on IC and FC systems was investigated. According to *t*-test results, immobilization had an insignificant effect on PHE biodegradation up to 1000 mg PHE (kg dry soil)$^{-1}$. However, less accumulation of IMs was observed at higher PHE content of soil for IC as compared to FC system, showing the superiority of immobilization. TOC analysis also showed the oxidation of PHE in soil samples into nontoxic intermediate metabolites that in turn were mineralized by our microbial consortium. Maximum PHE biodegradation was obtained at 30 °C, pH of 7, initial 2000 (mg PHE)(kg dry soil)$^{-1}$ and 600 mg L^{-1} of inoculum for both the systems. However, it was shown that the IC system was tolerable to acidic condition with retaining 85% of its biodegradation capability at neutral pH while the FC system was highly affected by acidic pH and retained only 33% of its capability. Our results show the superiority of cell immobilization compared to free cell system, especially at high concentration of PHE and under harsh environmental conditions. This can have generic application for other PAHs.

Key words Bioremediation, Immobilized cell, Intermediate metabolites, Slurry phase, Phenantherene

1 Introduction

Environmental contamination due to the industrial activities has caused detrimental effects on various ecosystems worldwide [1–3]. Polycyclic aromatic hydrocarbons (PAHs), ubiquitously present in

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natural environments, were perhaps the first recognized environmental carcinogens of which 16 compounds are listed as priority pollutants by the U.S. environmental protection agency (US EPA) [4]. Due to their low solubility and hydrophobic nature, they can easily be accumulated in soil where they could not be easily degraded under natural conditions. This necessitates the exploitation of various physico-chemical and microbial methods to safely remove these compounds from soil [3, 4]. Owing to the adaptability of microorganisms, bioremediation has been sought by many researchers for remediation of contaminated soils and this has also become a promising technique for the removal of PAHs [3–6]. Lu et al. (2011) in their review reported over 102 publications on PAHs biodegradation in soil and sediment [3]. A major problem in soil bioremediation is however the limited bioavailability of many contaminants due to their tendency to be adsorbed on fine particle fraction of soils such as clay (mostly a particle size of nearly 5 μm) [7–11] making up a significant fraction of most soils [8, 12]. Therefore, exploitation of competent techniques is essential in the removal of these adsorbed contaminants.

Since bioremediation in solid phase suffers from the long operational periods and low degradation rates, the simple and economical slurry phase method has drawn the attention of some researchers for treating PAHs contaminated soils [13–22]. Effective mixing, practical aeration, and enhanced contact of contaminants and microorganisms, which can result in an increased degradation rate, in addition to cost-effectiveness are some advantages of this technique [23, 24]. Due to the complexity of cell-environment interactions, several factors including temperature, oxygen content, pH, and salinity can affect bioremediation [3]. Some researchers have studied the effect of environmental conditions on PHE biodegradation in aqueous [25–28] and soil [10, 29, 30] phases using free cell (FC) system. Their results however show that extremes of temperature and pH, especially acidic pH condition, significantly decrease PHE biodegradation [10, 26, 28]. Therefore to exploit the advantages of bioremediation, it is necessary to retain the optimum conditions despite alterations in natural environments [1, 3–5, 31, 32].

To alleviate these adverse effects and to maintain the high bioremediation efficiency over long periods of time, cell immobilization has been employed in PHE biodegradation by some researchers [33, 34]. Additionally, immobilized cell (IC) systems are generally preferred to free cell systems due to their easier separation, possible reutilization of microorganisms, and preclusion of cell washout at high dilution rates [35]. Table 1 illustrates previous studies on PAHs biodegradation by cell attachment on solid surfaces and cell entrapment/encapsulation methods in soil phase.

Table 1
Biodegradation studies on PAHs using immobilization techniques in soil phase

Technique	Contaminant	Carrier	Microorganism	Ref.
Binding on solid surface	Phenanthrene, Pyrene	Plant residues or biochars	*Pseudomonas putida*, Indigenous bacterium	[36]
	Pyrene	Corn cobs	Different bacteria	[37]
Entrapment	Phenanthrene	Alginate	*Pseudomonas* sp. NGK1	[38, 39]
	Phenanthrene, Pyrene	BPVA	*Zoogloea* sp.	[22]
	Pyrene	PVA	*Candida tropicalis*	[40]
	Phenanthrene	Alginate	*Pseudomonas* sp. UG14Lr	[41]
	Pyrene	PVA-cinder	*Pseudomonas taiwanensis* PYR1 and *Acinetobacter baumannii* INP1	[42]

As can be seen only a few studies were on bioremediation of PHE contaminated soils/slurry using immobilized cell system [22, 37–41]. Furthermore, apart from a survey carried out in slurry phase by Li et al. (2005) at various low concentrations of phenanthrene and pyrene, the effect of environmental conditions (i.e., temperature, pH, and soil PAHs content) on PAHs biodegradation by the immobilized cell system in soil has not yet been investigated [22].

A common characteristic of catabolic pathways of polycyclic aromatic hydrocarbon such as PAH is the formation/accumulation of intermediate metabolites [43]. Nontoxic accumulated metabolites could further be taken up by cells; however, some metabolites could be inhibitory to cell growth when accumulated in the culture. Within PHE biodegradation pathway, hydroxylated aromatic compounds are known as the majority group of the intermediate metabolites (IMs) [43]. Several reports exist on the decrease of biological activity due to the accumulation of IMs and the effect of their competitive inhibition or toxicity on PAH biodegradation [44, 45]. Despite much research carried out on PAH biodegradation, the release and accumulation of IMs has remained challenging due to their different effect on cell metabolism.

In this study, therefore, we examined and compared PHE biodegradation by immobilized and free cell systems in slurry phase under different environmental conditions such as acidic/alkaline pH and low/high temperature in a clayey soil at high initial PHE concentrations. Additionally, the release and potential toxicity of intermediates were investigated.

2 Materials and Methods

In this work, Kaolin (SZWNK1 type) obtained from Iran China Clay Industries Corporation (ICCIC) was used as a clayey soil containing no organic carbon [8]. It consisted of (in w/w%): Al_2O_3, 24–25; SiO_2, 61–62; Fe_2O_3, 0.45–0.65; TiO_2, 0.04;

CaO, 1.2–1.5; MgO, 0.6; Na_2O, 0.3 and K_2O, 0.4 having a loss of weight on an ignition value of 9.5–10 (%, w/w). Soil was sieved, through a mesh size of 100 to obtain particles with sizes smaller than 150 μm, and stored in the dark at ambient temperature. This soil was then artificially spiked with PHE to prepare the contaminated soil samples. This was performed by dissolving 25, 50, 100, or 200 mg PHE in 100 mL n-hexane and addition of this solution to 100 g soil followed by sonication in an ultrasonic homogenizer for 10 min. Soil samples were then dried at 30 °C for 48 h and aged for 30 days at ambient temperature (30 ± 2 °C) before use. Examination of the PHE content of soil samples showed insignificant loss of PHE due to volatilization during soil spiking and drying. This is consistent with the findings of Park et al. (1990) reporting naphthalene to be the only PAH exhibiting volatilization from soils while a loss of less than 0.1% was observed for others [46].

A PHE-degrading microbial consortium obtained by screening and acclimatization of activated sludge taken from Tehran Oil Refinery was used throughout this study. The mineral salt (MS) medium for biodegradation experiments contained (in g L^{-1}) KH_2PO_4, 4; Na_2HPO_4, 5; NH_4NO_3, 4.0; $MgSO_4{\cdot}7H_2O$, 0.2; $FeSO_4{\cdot}7H_2O$, 0.05; $CaCl_2$, 0.001 and $MnSO_4{\cdot}7H_2O$, 0.02 in distilled water and the pH was adjusted to 7. Phenanthrene and PVA (with an average molecular weight of 146kD) were obtained from Merck and were used as the model contaminant and synthetic polymer for immobilization of microbial culture, respectively.

Microbial consortium kept in the fridge at 4 °C was first aerated for 2 days in the MS medium to prepare an active microbial suspension. For FC systems, slurry phase was inoculated with this cell suspension to obtain an initial cell concentration of 300 or 600 mg L^{-1}. For the IC system, microbial consortium was immobilized in PVA beads by the physical freeze-thaw method, as previously described by Lozinsky et al. [47]. In this study, desired numbers of PVA beads at a cell density of 5 mg mL^{-1} were used in slurry phase experiments using immobilized cells.

PHE biodegradation experiments by freely suspended and immobilized cells were performed in a 100 mL shake flask containing 16 mL of MS medium and 4 g of PHE contaminated soil. Since a range of 1:2–1:5 for a soil:water ratio has been reported to provide appropriate bioavailability and biodegradation of contaminant [18], a soil:water ratio of 1:4 was selected in our slurry phase experiments. After inoculation or addition of immobilized cell beads, the flasks were incubated at 30 °C and 200 rpm for 10 days on a rotary shaker.

The effect of initial PHE content of soil on biodegradation and formation of intermediate metabolites were separately investigated in slurry phase FC and IC systems at 250, 500, 1000, and 2000 (mg PHE)(kg dry soil)$^{-1}$. Additionally, the effects of various pHs (5, 7, 9) and temperatures (10, 20, 30, 40 °C) on PHE

biodegradation using FC and IC systems were investigated at initial 2000 (mg PHE)(kg dry soil)$^{-1}$ and inoculum size of 600 mg L^{-1}.

All the experiments were performed in duplicate and the average results were reported. To examine the non-biological PHE removal for the FC and IC systems, two control experiments void of cells at initial 2000 (mg PHE)(kg dry soil)$^{-1}$ were carried out which resulted in 8 and 11% of PHE removal, respectively.

To determine PHE biodegradation, the residual PHE concentration was examined by the extraction of PHE from the whole slurry sample using n-hexane, though aqueous phase is reported to contain insignificant amount of PHE [15]. Extraction of PHE was carried out by the addition of 10 mL n-hexane to suitable amount of slurry containing 1 g soil using the sonication method in an ice bath for 3 min followed by centrifugation at 3000 rpm for 10 min [15, 48]. PHE concentration in n-hexane was then measured by GC (Dani, Master GC, Italy), using fused silica capillary column (CP Sil 8 CB, 30 m × 0.25 mm, I.D., 0.25 μm, Varian, Agilent). The oven temperature was programmed as follows: hold 65 °C, 1 min; ramp rates 10 °C min^{-1} up to 140 °C and 25 °C min^{-1} to 250 °C, hold 5 min. The injection volume was 1 μL and the injector and detector temperatures were 240 and 300 °C, respectively. Nitrogen was used as a carrier gas at a flow rate of 1.5 mL min^{-1} [25, 44]. Efficiency of PHE extraction was examined by performing control experiments (without cells) at various PHE concentrations where an average PHE recovery of 97% was observed.

Hydroxylated aromatic compounds in the aqueous phase was measured by Folin-Ciocalteau reagent [49] and reported as IMs in this study [44, 50]. Following to the growth of microbial consortium on PHE, slurry samples were taken from both the FC and IC systems at different time intervals. These samples were immediately centrifuged at 3000 rpm for 10 min. To 2.5 mL supernatant, 0.125 mL Folin-Ciocalteu phenol reagent and 0.375 mL of 200 g L^{-1} Na_2CO_3 were added. The mixture was kept for 1 h at room temperature and the absorbance at 750 nm was read using spectrophotometer (Cecil BioQuest CE 2501). Resorcinol was used as the reference hydroxylated aromatic compound in soil [44]. Quantification of resorcinol equivalent (RE) was performed by using standard curve for 0–5 ppm resorcinol dissolved in distilled water.

To consider all remaining organic compounds in slurry samples during biodegradation experiments, the total organic carbon (TOC) of soil and chemical oxygen demand (COD) of aqueous phase were measured in addition to the measurement of PHE. These measurements allow investigation of possible biotransformation of PHE into other PHE oxidation products that are reluctant to further biodegradation and in some cases could be more toxic than the parent pollutants. A prior to analyses, slurry samples were

centrifuged at 4000 rpm for 10 min to separate the soil and aqueous phases. Chromic acid oxidation of Walkley and Black was used to assess soil TOC [51]. COD of aqueous phase was analyzed according to Standard Methods [52].

Average of phenanthrene biodegradation and intermediate metabolites formation in both the FC and IC systems were examined using statistical significance with the two-tailed *t*-test. All the experiments were performed in duplicate and the average results were used in statistical procedures.

3 Results and Discussions

Although enhancements have been previously reported in removal efficiency of petroleum hydrocarbon compounds by immobilized cells in aqueous phase at high concentrations as well as undesirable pH and temperature as given in Table 2, the effect of environmental conditions on PAHs biodegradation by IC system in slurry phase has not yet been investigated. Therefore, experiments were carried out to examine the effect of initial PHE content of soil and inoculum size as well as different temperatures and pHs on PHE biodegradation in slurry phase by immobilized cells in PVA beads.

Effect of inoculum size on PHE biodegradation in slurry phase at various initial soil PHE contents for the FC and IC systems is illustrated in Fig. 1.

Results at the lower inoculum size of 300 mg L^{-1} show incomplete PHE degradations for both systems at above initial 1000 (mg PHE)(kg dry soil)$^{-1}$. However at 600 mg L^{-1} inoculation, more

Table 2
Biodegradation studies on the effect of environmental conditions on removal of petroleum hydrocarbons using immobilized cell systems

Phase	Variable	Range	Pollutant	Matrix	Ref.
Aqueous	pH	4–11	2-Nitrotoluene	PUF	[53]
		4.5–9.5	Crude oil	Cotton fiber	[54]
	Temp. (°C)	16, 30	Naphthalene	Alginate	[55]
		20–45	2-Nitrotoluene	PUF	[53]
	Initial conc.	0.5–2.5% w/v	Crude oil	Agar, alginate	[56]
		50–800 mg/L	Quinoline	Alginate	[57]
		25–75 mM	Naphthalene	Alginate	[58]
		50–600 mg/L	Gasoline	Gellan gum	[59]
		15–30 mM	2-Nitrotoluene	PUF	[53]
Slurry	pH Temp. (°C) Initial conc.	5–9 10–40 250–2000 mg/L	Phenanthrene	PVA	Present work

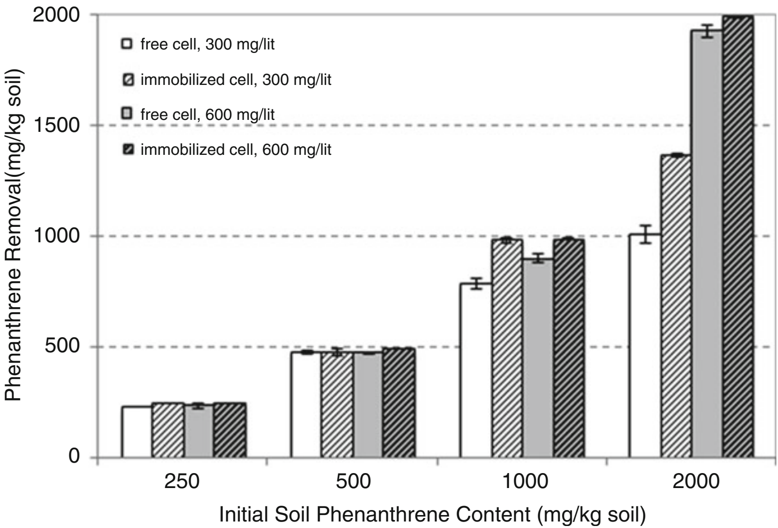

Fig. 1 Effect of initial soil phenanthrene content (250–2000 mg (kg dry soil)$^{-1}$) and inoculum size (300 and 600 mg L^{-1}) on phenanthrene removal by free and immobilized cells

than 95% of PHE was biodegraded at up to the high soil PHE content of 2000 mg (kg dry soil)$^{-1}$. According to the *t*-test analysis, average of PHE biodegradation (obtained from the FC and IC systems at different initial concentrations) insignificantly increased with increasing inoculum size (level of significance 0.05). This can be attributed to the low solubility of PHE in aqueous phase resulting in mass transfer limitation [60, 61]. However in slurry phase, contaminant is distributed within the soil matrix and is gradually transferred from soil and solubilized/emulsified in aqueous phase before it can be taken up by cells [62, 63]. Several researchers have pointed out the significant effect of inoculum size on PAHs biodegradation in soil [29, 64, 65]. Leys et al. (2005) reported enhanced anthracene and pyrene biodegradation at higher inoculum sizes of *Mycobacterium* sp. LB501T and VM552, respectively; however, they attributed this to the decreased lag phase in the slurry phase system [64].

Results for FC system at 300 mg (mL)$^{-1}$ inoculation given in Fig. 1 elucidate that the percentage of PHE biodegradation reduces from 100 to 50% with initial PHE content of soil, though this was alleviated by using a higher inoculum size. For IC systems at both inoculum sizes, complete biodegradation was achieved at up to an initial soil PHE content of 1000 mg (kg dry soil)$^{-1}$ and thereafter reduction to about 70% occurred at the lower inoculum size while it remained at 100% for the higher size of inoculum. Although enhanced biodegradation of PHE was observed in slurry phase system, according to *t*-test results, FC and IC systems show similar PHE removal efficiencies at different initial soil PHE content (level of significance 0.05). The higher tolerance of immobilized cells to

Table 3
PAHs biodegradation studies in slurry/soil phase using immobilized cell system

Phase	Contaminant	Matrix[a]	Initial conc. (mg/kg soil)	Removal(%)	Duration (Day)	Ref.
Solid	Phenanthrene	Alginate	100	40–100	60	[41]
Slurry						[22]
	Phenanthrene	BPVA	10–200	69–86	5	
	Pyrene			56–74		
	Pyrene	FTPVA	100	100	4	[40]
	Phenanthrene	FTPVA	250	100	10	Present work
			500	100		
			1000	99		
			2000	99.7		

[a]BPVA: Boric acid-PVA; FTPVA: Freezing and thawing of PVA

toxic compounds in aqueous phase has been previously reported for naphthalene [55, 58], crude oil [56], and PHE [60, 66]. This tolerance was also observed in slurry phase at initial 100 (mg PHE)(kg dry soil)$^{-1}$ by Li et al. [22], where immobilized *Zoogloea* sp. in PVA-alginate beads showed 84.9% PHE biodegradation compared to 37.3% with freely suspended cells.

Table 3 demonstrates previous studies on PHE biodegradation in solid/slurry phase by immobilized systems. As can be seen, solid phase bioremediation requires a long bioremediation time, while slurry phase bioremediation can be carried out in a much shorter period of time.

Table 3 also shows that the previous slurry phase studies were mostly at initial soil PHE content of lower than 200 mg (kg soil)$^{-1}$. Therefore in the slurry phase, no previous results were available at as high PHE concentrations of 2000 mg (kg soil)$^{-1}$ we examined in this work to compare our results. At low soil PHE content of 250 mg (kg soil)$^{-1}$, we achieved complete PHE removal using immobilized cells in PVA beads prepared by the freeze thaw method, while Li et al. [22] reported a removal of 75.4% at a lower initial 100 (mg PHE)(kg soil)$^{-1}$.

To ensure complete pollutant detoxification from soil [1], it is necessary to study the removal of not only the parent pollutant but also the accumulated intermediate metabolites (IMs) produced within microbial degradation pathways [44, 50]. Therefore, in this study, the time courses of IMs formation in slurry phase were examined at inoculum sizes of 300 and 600 mg L^{-1} and initial 250, 500, 1000, and 2000 (mg PHE)(kg dry soil)$^{-1}$ using the FC and IC systems. Results given in Fig. 2(a–d) show that at the two lower soil PHE contents (250 and 500 (mg PHE)(kg dry soil)$^{-1}$), IMs concentration first rises and after reaching a maximum it diminishes over time and reaches to very low values by the end of cultivation

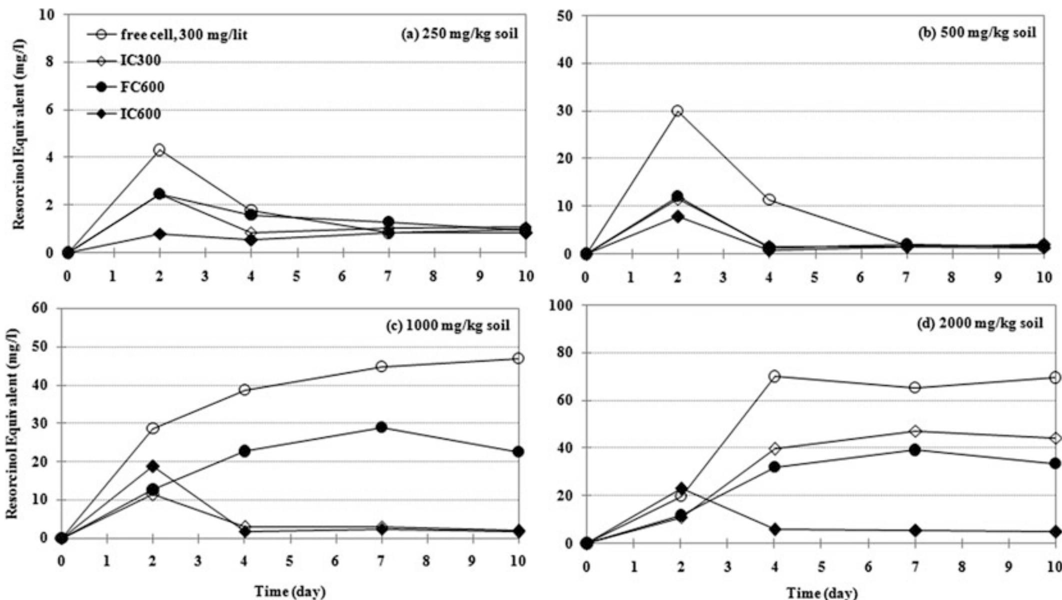

Fig. 2 Comparison of intermediate metabolites formation by free and immobilized cell (FC and IC) systems at soil phenanthrene contents of (**a**) 250, (**b**) 500, (**c**) 1000, and (**d**) 2000 mg (kg dry soil)$^{-1}$ as well as inoculum sizes of 300 (void) and 600 (filled) mg L^{-1}

for both FC and IC systems, though lower maximum values are observed for the IC system. This trend is similar to that observed by Silva et al. (2009) for PAHs contaminated soil [44]. For FC systems at 1000 (mg PHE)(kg dry soil)$^{-1}$ and 300 mg L^{-1} of inoculum, IMs concentration increases with time and finally reaches to 47 ppm (SD = 0.4), while at 600 mg L^{-1} inoculation, it goes through a maximum at 30 ppm (SD = 1.6) followed by a decline to 22 ppm (SD = 1.9) during the 10 days of incubation. For the IC system at 1000 (mg PHE)(kg dry soil)$^{-1}$, lower maximum IMs concentrations of 12 ppm (SD = 0.3) and 18 ppm (SD = 1.0) were achieved for 300 and 600 mg L^{-1} inoculation, respectively, after 2 days compared to those for the FC system. Similar trends were obtained at 2000 (mg PHE)(kg dry soil)$^{-1}$ for the FC and IC systems.

According to *t*-test results as shown in Table 4, we conclude that IC systems show enhanced removal of PHE intermediate metabolites, especially for polluted soils at 1000 and 2000 mg (kg dry soil)$^{-1}$.

High PHE removal of 96.6 was obtained in this study at an initial concentration of 2000 mg PHE/kg after 10 days for FC (see Fig. 1). This however shows the removal of only parent contaminant (PHE) form slurry phase and not its oxidized products (IMs). Therefore, we examined the hydroxylated aromatic compounds in aqueous phase of slurry phase as a representative of intermediate metabolites. Further to assess PHE mineralization/biotransformation

Table 4
***T*-test results comparing immobilized cells (IC) and free cells (FC) on intermediate metabolites formation**

Treatment(mg PHE/kg soil)		n	Mean	SEM	Calculated *t* value	Degree of freedom	P value	Are means significantly different? ($P < 0.05$)
250	FC	5	1.578	0.7379	1.280	8	0.2358	No
	IC	5	0.609	0.1630				
500	FC	5	8.954	5.619	1.157	8	0.2807	No
	IC	5	2.255	1.40				
1000	FC	5	47.65	12.81	3.218	8	0.0123	Yes
	IC	5	4.928	3.471				
2000	FC	5	44.98	14.06	2.440	8	0.0406	Yes
	IC	5	7.913	3.949				

Critical *t* value (alpha = 0.05): 2.31; SEM: Standard error of the mean

in the slurry FC system, soil TOC and aqueous phase COD were examined at initial 250–2000 mg PHE/kg soil and 600 mg/L inoculation as given in Fig. 3.

Similar trends of TOC (Fig. 3a) and PHE (Fig. 1) with time demonstrate the removal of both PHE and TOC from soil samples. Suitability of RE as an indication of IMs in the aqueous phase can be evidenced by similar trends of COD (Fig. 3b) and RE (Fig. 2). We can finally conclude that PHE in soil samples were oxidized into nontoxic intermediate metabolites that in turn were mineralized by our microbial consortium.

The effect of temperature on contaminant biodegradation is universally known to the researchers of environmental microbiology, with a nearly similar optimal temperature of 30 °C for PAH biodegradation [10, 25–28, 67]. Nonetheless, the aim of this work was to examine whether the immobilized cell system could tolerate the undesired temperatures in slurry phase bioremediation. To assess the effect of temperature (10–40 °C) on PHE biodegradation, slurry phase FC and IC experiments were carried out at 2000 (mg PHE)(kg dry soil)$^{-1}$.

Results given in Fig. 4a demonstrate an optimal temperature of 30 °C with more than 97% of PHE removal. As can be seen in Fig. 4a, both systems similarly lost their performances at 10 °C due to using a non-optimal temperature. Compared to the optimal PHE removal, a slight decrease at 20 °C and a sharp decrease at 40 °C were observed, though the IC system showed milder deviations from its optimal value. Effect of temperature at 20–40 °C on PHE biodegradation has been previously investigated in aqueous phase [25–27, 67] and soil phase [10, 28] using free cell system where the maximum PHE degradation was achieved at 30 °C. Kim et al. (2005) reported complete PHE removal from polluted soil at

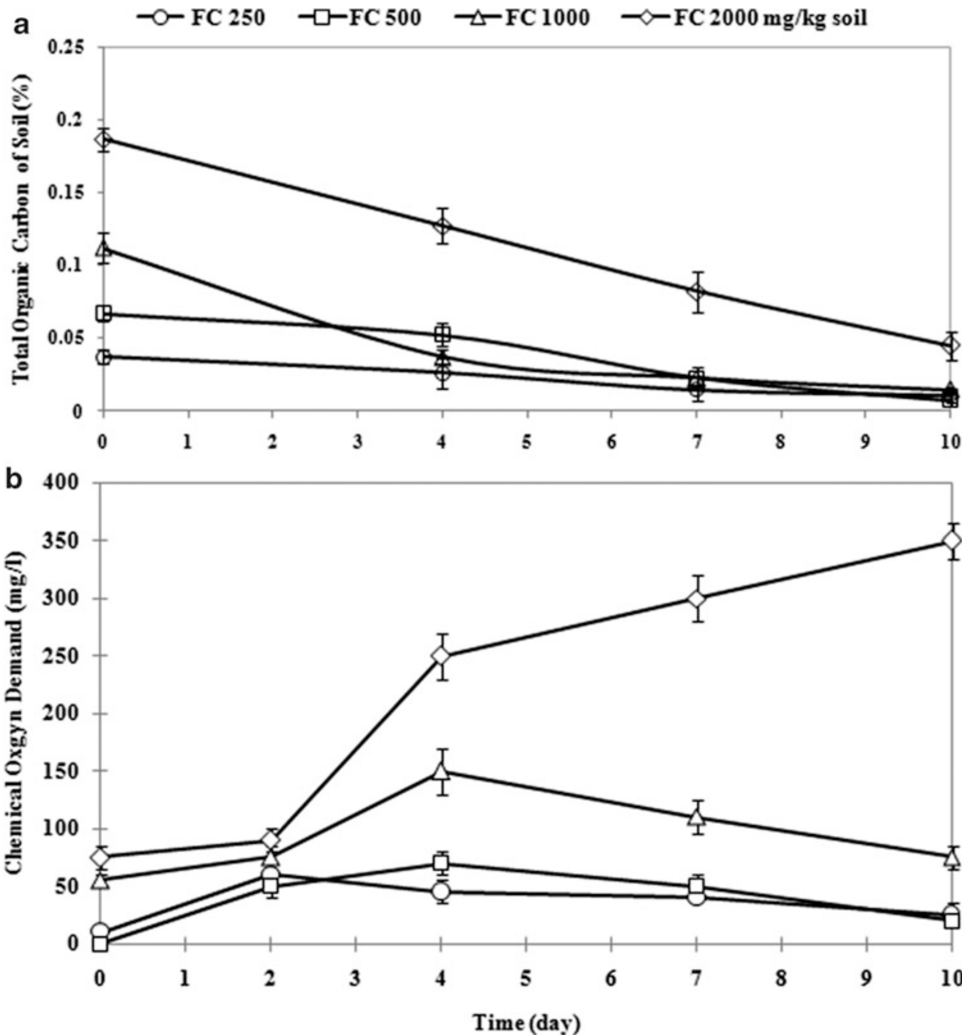

Fig. 3 Soil organic carbon content (**a**) chemical oxygen demand of aqueous phase (**b**) in phenanthrene biodegradation by free cell (FC) system at different concentrations in slurry phase

30 °C after 8 days, whereas 40 and 20% removals were obtained at 20 and 40 °C, respectively [26]. Using a microbial consortium in soil experiments, Yuan et al. (2002) also reported the optimal temperature of 30 °C for PHE biodegradation [10]. Slightly higher PHE removals for IC compared to FC systems at each temperature in this work are similar to that observed by Feijoo-Siota et al. (2008) for naphthalene biodegradation in aqueous phase [55]. Immobilized systems are usually preferred to free cell systems due to the cell protection under inappropriate environmental conditions [68].

It is generally known that neutral pH is favorable for growth and activity of most microorganisms and hence optimal pollutant biodegradation is expected to occur at pH of 7. However to assess

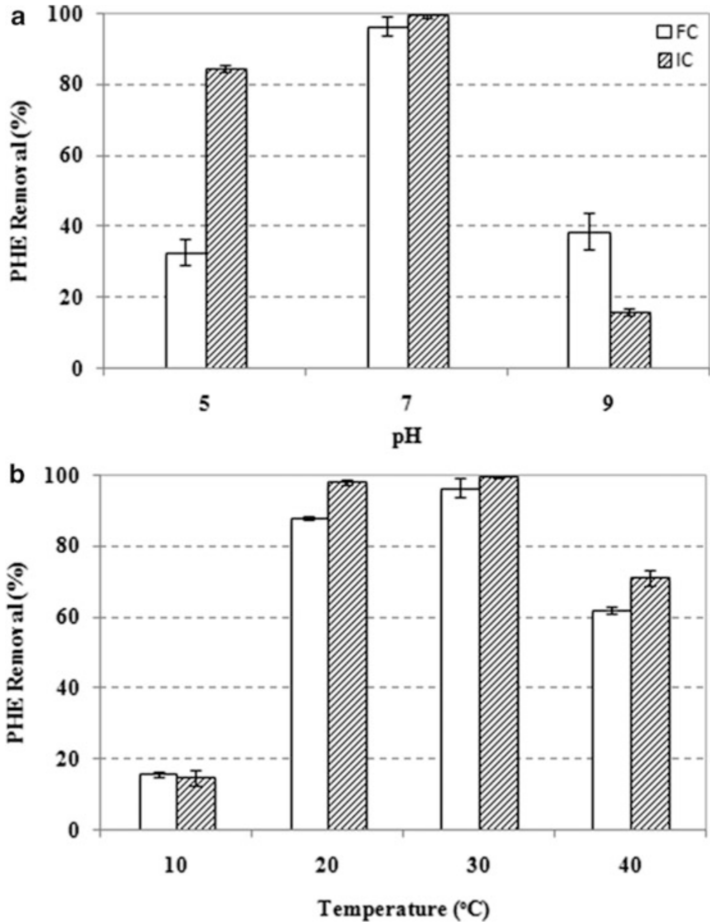

Fig. 4 Effect of pH (**a**) various temperatures (**b**) on free cell (*white*) and immobilized cell (hachure) systems performance at initial soil phenanthrene content of 2000 mg (kg dry soil)$^{-1}$ and inoculum size of 600 mg L^{-1}

the tolerance of FC and IC systems at acidic and alkaline pHs, the effect of initial pHs of 5 and 9 in addition to 7 on PHE biodegradation at initial 2000 (mg PHE)(kg dry soil)$^{-1}$ was investigated in slurry phase, the results of which are given in Fig. 4b.

For free cells system, biodegradation was highly affected by pH, with an optimal pH of 7. At both acidic and alkaline pHs reductions were observed, though system performance was more sensitive to alkaline condition. Although for both the IC and FC systems neutral pH was optimal, at acidic pH immobilized cell system showed the superior biodegradation of 85% compared to 33% for the free cell system. As shown in Table 5, some researchers also reported a drastic effect of pH on PHE biodegradation by FC systems at acidic pH in the aqueous and solid phases. Previous studies carried out on the effect of pH on biodegradation of five PAHs in soil under aerobic [10] and anaerobic [28] conditions have also shown that the maximum biodegradation constant (k)

Table 5
Effect of initial culture pH on PAHs biodegradation using free cell system

Phase	Pollutant	Initial conc. (ppm)	pH	Removal (%)	Duration (day)	Microorganism	Ref.
Aqueous	Phenanthrene	25	5	83	6	Isolated microorganism	[26]
			7	100			
			9	94			
		500	6	22	5	*Janibacter anophelis* JY11	[25]
			7	98			
			8	11			
		250	6	27	8	*Pseudomonas stutzeri* ZP2	[27]
			7	100			
			8	100			
Soil	Anthracene	25 mg/kg soil	5.2	12	63	*Sphingomonas paucimobilis* BA2	[29]
			7	100			

in each case corresponds to the neutral pH in FC. The superiority of cell immobilization under harsh environmental conditions has been confirmed in a review by Mrozik and Piotrowska-Seget [33]. Mulla et al. (2013) reported similar 2-nitrotoluene biodegradation by PUF-immobilized and free cell systems at neutral pH, while their IC system could abide pH changes [53]. Lin et al. (2014) obtained similar crude oil biodegradation using bacteria-immobilized cotton fibers and free cells at a pH range of 5.6–8.6, while the performance of the IC system was superior at pHs below 5 [54]. Nonetheless, the effect of pH has not been investigated in the few studies carried out on PHE biodegradation by immobilized cells in soils/slurry systems [22, 37–41].

As can be seen, our PVA-IC system was much less effective at alkaline compared to acidic pHs in the slurry phase. Similarly, Wang et al. (2005) observed this inferior performance of PVA encapsulated bacteria at alkaline pH of 9 for urea removal in aqueous phase [69]. Noteworthy to say that some immobilized cell systems were reported to tolerate both alkaline and acidic pHs, e.g., PUF-immobilized and bacteria-immobilized cotton fibers used by Mulla et al. [53] and Lin et al. [54], respectively. Since PAH contaminated soils are frequently acidic [70], we suggest the PVA-IC system as an alternative to the free cell system for PAHs biodegradation in soils/slurry phase.

4 Conclusion

Slurry phase bioremediation of the recalcitrant clayey soil showed that the microbial consortium used in this study was well capable of biodegrading phenanthrene at as high concentration as 2000 (mg

PHE)(kg dry soil)$^{-1}$ as sole carbon and energy sources. Although both free and immobilized cell systems degraded similar amounts of PHE at different initial soil PHE contents and optimal conditions, enhanced PHE biodegradation was achieved using immobilized systems under acidic condition. Moreover, less accumulationPhenantherene (PHE)Polycyclic aromatic hydrocarbons (PAHs) as well as enhanced biodegradation of intermediate metabolites of PHEPhenantherene (PHE)Polycyclic aromatic hydrocarbons (PAHs) biodegradation pathway were observed for IC compared to FC systems. This study therefore suggests PVA cryogel beads as an alternative to free cell system for slurry phase biodegradation of PAHs contaminated soils at high concentrations of pollutant and undesired environments, especially acidic soils.

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Chapter 7

Calorimetry and Soil Biodegradation: Experimental Procedures and Thermodynamic Models

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Abstract

Calorimetry measures the heat flow of any chemical, physical, and biological reaction and it is considered an important tool in all those scientific disciplines. Calorimetry evolves and focuses on designing more and more sensitive instruments capable of monitoring the heat rate associated with practically all living systems including soil. To study soil, there are sorts of calorimeters designed as multichannel systems that can monitor up to 24 samples at the same time. Calorimetry detects the heat released by the microbial metabolism, using low quantities of soil for the experimental measurements, ranging now from 0.8 g to 5 g depending on the goal of the study, on the kind of soil, and also on the calorimeter type. Beyond this, calorimeters permit monitoring the soil microbial metabolism directly and continuously, without disturbing the sample during long periods of time, and without the need to culture organisms from the soil or to add radiolabeled or fluorescent substrates.

All these advantages promoted the opening of new research goals in soil science to improve the existing knowledge about soil microbial metabolism, by searching and applying alternative indicators of the soil microbial biochemistry that can be quantified by calorimetry. More recently, and as a consequence of the latest advances in the design of these instruments, studies to monitor the response of the soil microbial population to changing temperature are starting to be considered, due to the direct involvement of soil in the environmental impact of climate change.

This chapter aims to give a specific and detailed description about how to apply calorimetry to study the soil microbial metabolism, responsible for soil biodegradation and deeply involved in the global C cycle, with guidelines going from the experimental design to the application of thermodynamic models to study the soil microbial biochemistry.

Key words Soil, Microbial metabolism, Calorimetry, Calorespirometry, Microbial metabolic efficiency, Calorespirometric ratios, Temperature

1 Introduction

Soil is the most important primary resource on earth, together with water. It is constituted by a mix of inorganic, mineral, and organic material forming complex macromolecules where microorganisms form a basic part of it. Soil is the natural habitat for a vast microbial population that consists mainly of bacteria, actinomycetes, fungi,

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protozoa, and algae. The microbial population and the organic matter are responsible for the soil fertility. In a global environmental stage where human population increases at a rate that threatens the sustainable use of natural resources, to keep soil fertility becomes of upmost importance to guarantee human food supply [1].

The soil microbial metabolism plays an essential role in the global carbon (C) cycle. It is responsible for the biodegradation of organic C sources that feed the soil organic fraction, and for the parallel release of nutrients for plant growing. These biodegradation processes take place through biochemical paths that release CO_2 to the atmosphere. The CO_2 contributes to global warming and for that reason soil exploitation must preserve the natural organic matter and must minimize the impact of CO_2 on the atmosphere. The last is a challenging goal because of the lack of knowledge about the soil microbial structure and composition, about the chemical composition of the soil, about the metabolic functions of a certain soil microbial structure, and about the interconnection of all these mentioned factors [2]. As an example, we cannot contribute to develop soil management strategies that preserve the natural soil carbon, if we do not understand and if we cannot measure, the microbial metabolic paths that diminish the loss of that carbon through CO_2 release. To achieve this challenging goal, the first necessary step is to provide the right methodologies to measure the soil microbial activity.

Right now there are a remarkable number of different methodological options to apply in soil science to get information about the soil microbial structure and composition [3], about the soil biodegradation rates [4, 5], and about the soil organic matter composition [6], but there are not many methodological options to measure the connection among the soil microbial structure and composition, the microbial metabolic functions of that structure, and the soil organic matter properties, as well as the role of these interconnections in the soil capacity to keep the organic C [7]. If we need to preserve the natural capacity of the soil to keep the organic C, it is essential to measure that capacity in some way, and that involves the ability of the microbial metabolism to keep it or to lose it through CO_2. There are not many methods allowing us to obtain that information, and for that reason the soil microbial metabolic efficiency (SMME) to use C is poorly investigated. SMME is defined as the energy or C quantity that microorganisms can keep from the external inputs as microbial biomass. As a consequence, the contribution of these small scale processes taking place in soil to the global C balances are not considered by the current models employed, despite the SMME being essential for both: keeping soil carbon and controlling the loss of that carbon as CO_2, which constitutes the real nucleus of the global C cycle process. There are in the literature different attempts to quantify that efficiency focusing on the quantification of the CO_2

release by microbial respiration [8]. But the rate of CO_2 dissipation by microbial metabolism is very sensitive to many factors, such as the soil environmental conditions, the water content of the soil samples, the temperature at which those measurements are done, as well as all the procedures involved in the preparation of the soil samples for those measurements. These features affect the development of models to quantify the SMME [9].

CO_2 is one of the products of the soil microbial metabolism but not the only one. Most of the metabolic processes release heat too, and the microbial metabolic rate can be measured also by the heat rate of those reactions. The last can be easily done by calorimetry.

Calorimetry measures the heat rate of every reaction with instruments that provide higher sensitivity than the conventional methods used to measure the CO_2 released by the soil microbial metabolism. Calorimetry measures the heat rate directly, continuously and in real time without disturbing the soil sample, and could give more realistic information of the soil microbial metabolism than by the CO_2 rates alone. This potential of calorimetry to be applied in soil science is not new and the literature shows the effort to develop this method to study soil microbiology since 1973 [10–12].

It has been recently stated [13] that the heat rate of the soil microbial metabolism is more stable than the CO_2 rate, and for that reason, measuring heat and CO_2 dissipation might be a more robust way to characterize microbial activity than CO_2 production alone. Since these both parameters may not evolve in the same way under different environmental factors and soil managements, the use of both as indicators of microbial metabolism has the potential to increase our knowledge about soil biochemistry. Therefore, calorimetric methods stand up now as an innovative option in environmental microbiology.

Calorimetry and calorimeters are adapted now for measuring both, the heat rate and the CO_2 rate of the microbial metabolism by different procedures. This feature introduces new options for quantifying and interpreting metabolic indicators for soil biodegradation that had been never applied before in soil science. The measurement of the heat rate of a microbial reaction launches some thermodynamic models to quantify the SMME and bring alternatives to link the soil microbial degradation rates with the nature of the substrates used by soil microorganisms.

The calorimetric devices are highly versatile allowing the study of a wide variety of processes in soils [14, 15], but this chapter will focus on those applications that have been better explored and are more extended in soil microbiology, as well as on those showing up with the design of new calorimeters.

2 Application of Calorimetry to Monitor the Biodegradation of External C Sources in Soil

The measurement of the soil microbial response to the addition of external C sources gives information about the size of microbial biomass, about changes in community structure and functional diversity, and has been applied to a wide range of soil habitats undergoing changes in land use or disturbance due to pollution [16]. The most of them evaluate the capacity of a certain soil sample to respire a sole C source or a wide range of them by measuring the release of CO_2.

Calorimetry has been and can be applied with a similar goal to evaluate the same response based on the heat rate. The heat rate has the advantage to give a more global picture of the soil microbial metabolism because every metabolic process absorbs or releases heat, and calorimetry measures both, while CO_2 production is just linked to respiration through exothermic reactions. Therefore, when one measures the soil microbial metabolism by the heat rate, one is measuring the total metabolism in soil and not only the CO_2 dissipative paths. Calorimetry is especially useful too when it is applied to soils where the use of CO_2 limits the study of the soil microbial activity, such as soils with very low C content and very low biodegradation rates, and desert soils [17]. The development of techniques to measure the soil microbial response to a high sort of different substrates to yield a catabolic fingerprint of a soil sample [18], also opens new options where calorimetry can be valuable. Multichannel calorimeters would permit a similar evaluation with additional information about the kinetics of the degradation of those substrates.

This section of the chapter details the most adequate calorimetric procedures to evaluate the microbial response of soil microorganisms to external C sources and how it can be quantified.

2.1 Material and Methods

2.1.1 Calorimeters

TAM Air and TAM III-IV (TA Instruments) are the calorimeters more used for soil research. There are more types that can be used also but the advantage of TAM air and TAM IV relies in that both are multichannel systems. TAM Air has eight calorimetric channels allowing the measurement of eight samples at the same time, while TAM IV allows the measurement of 24. TAM IV also provides higher sensitivity and stability than TAM Air, but at a higher cost that makes TAM IV unaffordable for many soil scientists. TAM IV allows the 24 measurements with calorimetric ampoules of 4 ml while TAM air uses 20 ml vessels. Although the last is considered advantageous for environmental science, in the case of soil the use of 1 g or 5 g of sample does not involve any limitation to study a soil microbial process. In fact, using 5–10 g of sample with high water holding capacity (WHC) at the required water content in soil

microbiology can produce an intense evaporation inside the 20 ml vessels that may corrupt the measurement. For this reason, the protocols for sample preparation used by other methods in soil science may not be the most adequate for soil calorimetric experiments in some cases, but that does not constitute a limitation of the method by itself.

2.1.2 Soil Samples Preparation Before Calorimetric Measurements

Soil samples must be collected under certain sampling criteria that depend on the goal of the measurement. After being collected, they are usually sieved through 2 mm or 0.5 mm mesh size depending on the soil fraction to be studied. This is usually a necessary step performed by all the different methods involved in the study of the soil microbiology to make comparative studies among different soils. The mechanical sieving of the soil usually disturbs the microbial activity due to the release of single molecules from the organic matter (OM) [19] that can be assimilated by the soil microbial population. This perturbation is usually recorded as a high increase of both, CO_2 and heat rates, that may not represent the natural soil microbial activity. For this reason, it is important to let the soil stabilizes again for a certain period of time before the calorimetric measurements. It is possible to monitor the stabilization process introducing the samples in the calorimeter immediately after sieving, to obtain the time of the stabilization of the samples. Samples are considered to be stable when the heat rate is almost constant or declines slightly with time. If the samples cannot be measured immediately after stabilization, they must be stored inside polyethylene bags at 4 °C for 1–3 months or frozen at −21 °C according to the common protocols of soil microbiology [20]. Until now, calorimetry has been applied to soils stored at 4 °C for short periods of time (1–6 months) and there is no information yet about how the use of frozen samples affects the calorimetric measurements. The soil microbial heat rate decreases as time of storage increases yielding not reproducible results after 6 months stored at 4 °C [21].

Another factor affecting the extent of the soil microbial response to a certain substrate is the water content of the soil samples. Heat and CO_2 rates increase with increasing water content up to 60% of the soil water holding capacity (WHC). Therefore, for comparative studies of that response among different soils, it is important to bring all of them to the same water content. It is assumed that maximum rates are obtained at 60% of WHC; therefore, the water content of the samples must be brought close to that percentage [22]. This feature introduces some controversy about the right treatment of the samples to be brought to a certain water content. If the water content of the samples is higher than 60% of WHC at the moment of the sampling, it is necessary to dry them so that they can be rewetted to the water content desired, but drying and rewetting soil may affect the soil microbial population yielding metabolic rates that cannot be compared with those in nature [23].

Our experimental tests by calorimetry showed that drying and rewetting avoiding extremely low values of water content yielded satisfactory results because the heat rate is more stable than the CO_2 rates. Storing the soils at 4 °C at the water content they had in the moment of the sampling when it is close to 60% of WHC, depletes the heat rates strongly and makes difficult the activation of the microbial activity from 4 °C to the temperature of the calorimetric measurement (usually about 25 °C) in many samples. It is obtained better microbial activation when soils are stored at about 15% of the WHC and then rewetted to 50–60% of WHC for calorimetric measurements, but this means that soils must be air dried at about 20–21 °C after sampled for about 1–6 days depending on the initial water content. This treatment is not as extreme as the reported in the literature showing changes in the microbial activity attached to the drying conditions [23] because the drying conditions used for calorimetric measurements are those usually taking place in the natural environmental conditions of the soil.

2.1.3 Soil Samples Preparation for Calorimetric Measurements After Stored at 4 °C

Before calorimetric measurements, samples must stabilize at the temperature at which the calorimetric measurement will be done. The calorimetric monitoring of the soil microbial response to external C sources is usually done under isothermal conditions. Calorimeters usually allow these measurements at a wide range of temperatures, ranging from about 4 °C to 150 °C depending on the calorimeter type. Most of these measurements have been reported to be at 25 °C in the literature. The main reason for that, in our case, was the determination of the enthalpy for the glucose microbial degradation under standard conditions (25 °C and 1 atm of pressure) in order to compare the obtained values with the enthalpies of combustion for the glucose and other C substrates [24]. But it does not mean every calorimetric measurement of the microbial response to different substrates has to be done at that temperature.

The adaptation of the microbial metabolism from 4 °C to the temperature of the measurements is very fast and takes place in about 24–48 h.

To adjust the soil water content to 50–60% of WHC, it is necessary to rewet the samples at the water content desired after the adaptation to temperature. The rewetting causes in many soils an activation of the heat rate when the water content of the sample stored was about 15% of WHC or lower, and for that reason it is necessary to let the samples stabilize again. The stabilization time may depend on different factors, such as the soil sample size that will be introduced in the calorimeter. The easiest way to know how long it takes for samples to stabilize again after rewetting is introducing the sample in the calorimeter immediately after rewetting, to monitor the soil microbial response to the water addition. This

response, from our experience, is different depending on the soil. We have registered soils with not special response to the addition of water showing a stable heat rate immediately after the water addition, and soils that respond fast with an exponential increase in the heat rate that suggests a microbial growth reaction as a consequence of the water addition. The duration of this reaction may be variable too, but when using low quantities of sample, it usually does not take more than 48–96 h, although in some cases it can be found no initial response to the water addition and an exponential increase in the heat rate starting 72 h, or even later, after the water addition. For respirometric measurements, protocols recommend to let samples stabilize after rewetting for longer time periods, but those protocols do not measure the CO_2 rates during this stabilization time continuously, and for that reason it is difficult to know how stable is the soil microbial metabolism when the external substrates are added. By calorimetry the stabilization process is monitored continuously and the external C source is not added until a metabolic steady state is reached, which is very easy to demonstrate by the evolution of the soil microbial metabolic heat rate in the calorimetric plots. Once the duration of the reaction of the samples to water addition is known for the soils to be studied, the stabilization can be done outside the calorimeter, keeping the soil samples inside polyethylene bags with a water container to keep the soil water content reached.

Another factor is the quantity of soil used for the calorimetric measurement. Calorimeters have calorimetric vessels with different capacity ranging from 1 ml to 25 ml. The quantity of soil depends on that volume and on the sensitivity of the calorimeter. It is common in the literature to use 5 g of soil for 20 ml vessels [25] and from 0.8 g to 1.2 g of soil for 4 ml vessels [26, 27]. It is important to keep the right relation between soil mass and vessel volume to maintain the oxygen (O_2) supply that microorganisms need inside the vessel because it is sealed during the calorimetric measurement. Soil mass, vessel capacity, and the O_2 consumed by the microbial reaction that will be stimulated, must be controlled and designed adequately for the calorimetric experiments. That involves too the quantity of external substrates to be added so that O_2 inside the vessel does not limit the microbial degradation of those substrates, especially if the goal of the experiment is to quantify the SMME by some of the models reported in the literature.

It is possible to determine if the available O_2 inside the vessel, once closed for the calorimetric measurement, is enough to allow the oxidation of the C source added by the soil microorganisms. The O_2 demand for the complete aerobic oxidation of glucose to CO_2 and water is 6 mol O_2/mol Glucose [28]. It can be similarly determined for other C sources.

All those features must be checked before starting the experiments, in order to design the stabilization time that samples need, the quantity of soil sample, and the quantity of substrates that will be added to the soil samples. Once these factors are known, the protocol to follow can be summarized as follows when glucose is the external C source:

- Take soil subsamples from the soils stored at 4 °C that are going to be measured. The size of these subsamples depends on the quantity of soil to be introduced in the calorimeter. Get a size enough to make at least three replicates per soil. As an example, if you are going to use 4 ml calorimetric vessels, take a 10 g subsample to be prepared, to introduce in the calorimeter at least three aliquots of 1 g taken from the 10 g stable subsample.
- Put the soil subsamples in some kind of container inside polyethylene bags and let them stabilize during 24–48 h to the temperature at which the calorimetric measurement will be done.
- Bring the subsamples to the desired percentage of water content and let them stabilize again inside the polyethylene bags with a water container inside, during the period of time settled by the previous calorimetric experiments at the temperature of the measurement.
- Take the replicates to be introduced in the calorimetric vessels (5 g of the soil subsample for 20 ml vessels or about 1 g for 4 ml vessels). Make the necessary replicates to get reproducible results.
- Add to the replicates inside the calorimetric vessels the substrates. The quantity of the substrate must be adequate to the O_2 demand. For example, for 1 g of soil in the 4 ml calorimetric vessel, the available O_2 inside the vessel is enough for the aerobic oxidation of 1.2 mg of glucose that can be added as powder or into a solution of 0.2 ml of water to not alter the soil water content [27]. Adding glucose dissolved in water makes the glucose more available to microorganisms than as powder.
- Close the vessels and introduce them in the calorimeter for the measurements.

The proposed protocol is adequate for parallel measurements of the CO_2 rates. Calorimetry is very versatile and allows different preparations of the samples. Protocols can be adapted for other parallel measurements such as enzymatic activities.

2.1.4 Calorimetric Measurements

The most common calorimeters applied in soil microbiology are the heat conduction type. These calorimeters measure the metabolic activity in a living system as thermal power (ϕ) in microwatts (μW), under isothermal conditions and continuously, giving a plot

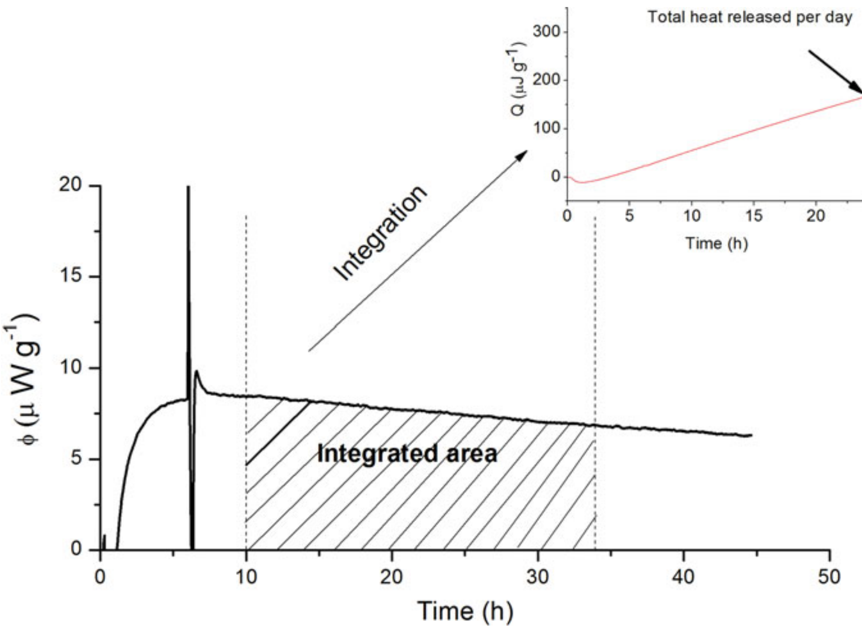

Fig. 1 $\phi - t$ plot of soil basal metabolism and the heat accumulated plot obtained after integration for 24 h. Vertical dashed lines show the initial and final limits of the integral starting once ϕ stabilizes after inserting the soil in the calorimeter

representing ϕ versus time (ϕ–t plot in Fig. 1). These plots permit tracking the evolution of the thermal power of most biological processes. The measurement principle of the thermal power by heat conduction calorimeters is designed as a differential signal between a sample and a reference. Depending on the calorimeter type, the reference can be an inert material that is part of the calorimeter, or can be an empty calorimetric vessel where a sample designed for the experiment is introduced as a reference. Therefore, when measuring a metabolic rate, it is necessary to make the correction of ϕ values from the measured sample with respect to the ϕ value from the reference sample applying Eq. 1:

$$\phi = \phi s - \phi r \tag{1}$$

where ϕ is the thermal power of the metabolic process being measured, ϕs is the thermal power of the measured sample, and ϕr is the thermal power of the reference sample.

In some calorimeters this step may not be necessary if the reference sample is integrated within the calorimetric design and the apparatus automatically corrects the signal of the measured sample.

The direct measurement of ϕ provides a measure of the rate of a reaction that, under the conditions inside the calorimeter, is

connected to the heat released by the reaction in Joules by the following equation:

$$\phi = dQ/dt \quad (2)$$

where *Q* is the heat in Joules dissipated or absorbed depending on the thermodynamic nature of the reaction (exothermic or endothermic). Therefore, Eq. 2 permits converting the thermal power of the reaction to the heat of the reaction, that is from microwatts (μW) to microjoules (μJ):

$$dQ = \phi dt \quad (3)$$

When applied to microbiology, Eq. 3 measures the metabolic rate and can be considered also a measure of the degradation rate of a substrate by microbial metabolism, giving useful information about soil biodegradation.

The temporal evolution of ϕ gives different profiles of the ϕ–t plots that can be attached to a certain microbial metabolic state [29]. Application of Eq. 3 may acquire different interpretations and applications depending on the metabolic state at which microorganisms are introduced in the calorimeter. These metabolic states can be growth, maintenance, and basal metabolism, although when applied to soil microorganisms, maintenance and basal metabolism cannot be differentiated. Therefore, the soil microbial metabolism monitored by calorimetry can be growth and basal metabolism, named basal respiration when measured by the CO_2 production rate [30].

These two states have been differentiated by calorimetry for soils, based on the observed evolution of the *ϕ–t plots.* Soil basal metabolism takes place at an almost constant or at a slight decline of ϕ with time (Fig. 1) and thus can be quantified by both, ϕ, or by the heat rate determined by Eq. 3. The first option provides a graphical picture of the soil basal metabolism evolution over time and the detection of any perturbation in that metabolic state. Application of Eq. 3 gives a quantitative value of the soil basal metabolic rate that can be useful in comparative studies involving different soil samples. Application of Eq. 3 for quantifying soil basal metabolism involves the integration of the ϕ–t plot within an interval of time that must be the same for all the soil samples (Fig. 1). Therefore, it can be useful to perform the integration of the plot for 24 h to give the heat rate (*Rq*) in Joules per gram of soil and day ($J.g^{-1}\ d^{-1}$) or to normalize the heat rate to the time interval used for the integration (24 h) to give *Rq* in Joules per gram of soil and hour ($J.g^{-1}\ h^{-1}$) [31]. Most of the software coupled with the calorimeters allows this conversion. *Rq* provides a quantitative measure of the soil basal metabolic rate that can be useful to be compared to the CO_2 rates or that can be normalized to the carbon content of the soil or to the soil microbial size as done with the CO_2 rates.

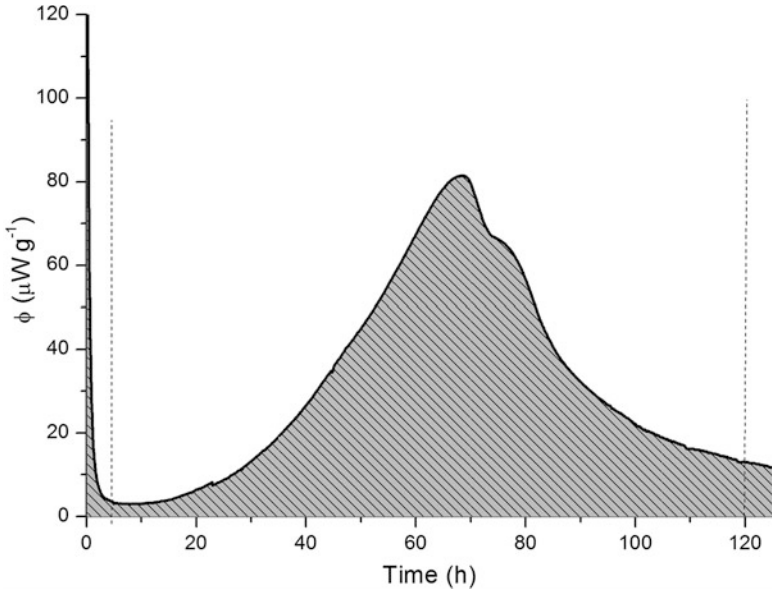

Fig. 2 Typical $\phi - t$ curve obtained after adding glucose to a soil sample. Vertical dashed lines show the limits of the integration. Shadowed area shows the integration is done with respect to zero as base line

Microbial growth, or a positive response of a microbial population to a certain substrate, is also detected by calorimetry by a change in the ϕ–t plot of the basal metabolism from a constant or slight temporal decline of ϕ to an exponential increase of ϕ with time (Fig. 2). The shapes of these curves vary depending on the type of microorganism growing or being activated [29] and it is considered to reflect different phases of the microbial growth reaction or of the microbial activated process caused by the substrate added. These ϕ–t plots are characterized by an initial lag phase taking place at a remarkable higher ϕ value than that of the basal metabolism. ϕ values in this lag phase have been associated with the microbial size being activated when the substrate added is glucose [32, 33]. The duration of this lag phase is variable and it is usually followed by an exponential increase in ϕ that has been associated with microbial growth in soil after glucose addition [27, 34]. This exponential increase has a variable duration depending on the soil and continues until reaching a stationary phase where ϕ keeps constant for a certain time before starting to decline again to a new basal metabolic state.

Application of Eq. 3 in this case yields the total heat (Q) released by the microbial reaction caused by the addition of an external substrate, in Joules per gram of soil, and it is not a rate as when determined for microbial basal metabolism, because the limits of the integral are different. In the case of microbial growth, the limits must be the initial time at which the activation starts and the

time at which the new basal state is reached, defining the initial and final state of the process (Fig. 2). When applied to basal metabolism there is not an initial and end but an interval of time that can be applied at your convenience and that define only the quantity of heat released along that interval of time. Therefore, the period of time must be introduced in the units defining Q in that case, converting Q in a rate Rq. That is not the case when Eq. 3 is applied to a process with a clear beginning and end in the ϕ–t plot. In this case, Q defines the "total heat released by that process or reaction" and introduction of time is not necessary unless we need to know how fast the whole reaction is. Although poorly explored in soil yet, the obtained Q values from different substrates added to soil could be a measure of the soil microbial sensitivity to different nutrient sources for catabolic fingerprints characterized by calorimetry [35].

Q released by microbial reactions caused by glucose addition to soil, is involved in the calculation of metabolic microbial efficiency, η. That is the percentage of energy from the glucose invested into microbial biomass formation, and the percentage that is released through the formation of CO_2 and water [36]. Quantifying η is possible for soils by calorimetry by different thermodynamic models, providing a measure of the carbon sequestration capacity of the soil microbial metabolism.

2.1.5 Thermodynamic Models to Quantify SMME

SMME can be determined by enthalpy models that associate the total heat released by the biodegradation reaction of the substrate added, Q, with the microbial biomass formed and with the quantity of that substrate that was degraded to CO_2 and water.

The main limitation is that to apply the enthalpy models, the reaction taking place in soil when adding the substrate must be modeled. The last was achieved for the soil microbial aerobic degradation of glucose and ammonium sulfate. The reaction can be written as follows:

$$a\ C_6H_{12}O_6 + b\ O_2 + c\ \mathrm{NH_4}^+ = \mathrm{CH}_{1.8}O_{0.5}N_{0.2} + d\ \mathrm{CO_2} + e\ H_2O + f\ H^+ \quad (4)$$

where $CH_{1.8}O_{0.5}N_{0.2}$ is the average chemical formulae for microbial biomass, and a, b, c, d, e, and f are the stoichiometric coefficients of the reaction. The enthalpy of the metabolic reaction 4 would yield SMME in terms of energy, η, and the determination of the stoichiometric coefficients would yield the SMME in terms of the quantity of C kept in the form of new microbial biomass, and the quantity lost as CO_2. The last is namely by other researchers as C use efficiency, CUE [37]. It has been experimentally demonstrated that the exponential increase in ϕ in soils after adding glucose is accompanied by microbial growth [27, 38]. Therefore, the glucose is oxidized to CO_2 and water but part of that energy is invested in the formation of new microbial biomass. The degree of coupling between the oxidative reaction to CO_2 and water and the

reactions involved in microbial biomass formation gives the metabolic efficiency, η. The degree of coupling, η, can be determined by Battley's equation [36]:

$$\eta = (\Delta_{\mathrm{NC}}H - \Delta_C H)/\Delta H_{\mathrm{NC}}H \quad (5)$$

where $\Delta_{NC}H$ is the enthalpy of combustion for the non-conservative reaction, that is in this case, the complete combustion of the glucose to CO_2 and water, -2810 kJ mol^{-1}, and $\Delta_C H$ the enthalpy for the conservative reaction, that is when the carbon source oxidation is coupled to a microbial growth reaction. $\Delta_C H$ is measured by calorimetry when added glucose and ammonium sulfate to the soil. This enthalpy can be experimentally determined by relating the total Q released by the reaction, obtained integrating the *ϕ–time* curve (Fig. 2), with the quantity of substrate consumed, giving Joules per mol of substrate degraded:

$$\Delta_r H_S = Q/[S] \quad (6)$$

where $\Delta_r H_S$ is the enthalpy for the conservative reaction, Q the total heat released, and S the quantity of substrate added degraded. Q can be related to the quantity of microbial biomass formed (ΔX) in Joules per mol of microbial biomass C:

$$\Delta_r H_x = Q/\Delta X \quad (7)$$

There are various attempts in the literature that have used the Battley's equation [36] to calculate η for soils using both, $\Delta_r H_x$ *and* $\Delta_r H_S$ [39, 40]. The use of $\Delta_r H_S$ involves the complete oxidation of the glucose added to the soil and that is not easy to achieve for every soil, making necessary parallel experimental analysis to evaluate the percentage of the substrate added used by the soil microbial population, complicating the experimental phases [27, 38]. Application based on $\Delta_r H_x$ needs that the microbial growth rate caused by glucose be determined, but calorimetry is commonly used in microbiology with that goal [14, 41] and therefore the whole calculation involves the same calorimetric experimental phase developed. To calculate $\Delta_r H_S$ from the experimental $\Delta_r H_x$ values also involves that the enthalpies of combustion of reactants and products in Eq. 4 be known. $\Delta_r H_x$ is defined by the following equation [39, 42]:

$$\Delta_r H_X = \left(\Delta_c H_{glucose}/\Upsilon_{X/S}\right) - \Delta_c H_X + 0.2\,\Delta_c H_N \quad (8)$$

$$\Delta_r H_X = \Delta_r H_{glucose}/\Upsilon_{X/S} \quad (9)$$

where $\Delta_c H_{\mathrm{glucose}}$ is the enthalpy of combustion for the glucose, $\Delta_c H_x$, is the enthalpy of combustion for the microbial biomass (-559 kJ mol^{-1}C) [42], $\Delta_c H_N$ the enthalpy of combustion for the ammonia, $\Upsilon_{X/S}$ is the growth yield of the reaction, that is the mol of microbial biomass synthesized per mol of substrate, and

$\Delta_r H_{glucose}$ is the enthalpy for the microbial substrate degradation reaction coupled to microbial growth that can be determined from the system formed by Eqs. 8 and 9.

Once these enthalpy values are known, the stoichiometric coefficients of Eq. 4 can be determined too by a mass balance.

Note that for an accurate calculation of the enthalpies, the correct integration of the ϕ–t plot is an essential step and that the following criteria for the integration must be stated:

– The beginning of the integral includes the lag phase and the end is the point of the ϕ–t curve reaching a new steady metabolic state (Fig. 2).
– The integral must be done respect to zero and not to a base line constructed with the initial and final ϕ values of the curve, because the heat released by the activation of the microbial growth reaction in the lag phase is involved in the efficiency of the process too.

3 Calorespirometry

Calorespirometry is the simultaneous measurement of the heat and CO_2 production rate of any reaction. It has been mainly applied to quantify the heat and CO_2 rates of metabolism in living systems from single whole organisms to cells and microorganisms. Application to soil is quite recent and still under development. To study the soil microbial metabolism by both heat and CO_2 rates may provide more information than that given by the CO_2 rate alone for different reasons. In soil, a vast variety of microorganisms degrade complex organic macromolecules that involve many different biochemical paths. Because the organic matter macromolecule is constituted by different fractions at different oxidation states, there are many metabolic paths running simultaneously. Most of them dissipate CO_2 but others do not, in particular those involving biodegradation of reduced substrates. For this reason and depending on the microbial fraction being active, the CO_2 and heat rate may not evolve in the same way. These differences in the CO_2 and heat production rates may be helpful to understand better the biochemistry of different soils. As an example, by the CO_2 rate we only know how fast or slow is the biodegradation of the soil organic matter, but measuring CO_2 and heat rates we can know if that biodegradation is done by heterotrophs, through aerobic or anaerobic metabolism or even if there are microbes using other electron acceptors than O_2. Also, in comparative studies, it could allow detecting changes in the soil organic matter nature used by the soil microbial population that could be attached to a certain ecosystem or soil management. When applied to microbial growth reactions, it also provides alternative models to quantify the SMME.

Heat and CO_2 rates of any biotic reaction can be measured by independent methods but calorimetry has the advantage to measure these rates simultaneously.

There are different procedures to perform those measurements by calorimetry but the most extended is the use of a CO_2 absorbent (NaOH solution) introduced in the calorimetric ampoule or calorimetric vessel where the biological sample is placed [43].

3.1 Material and Methods

3.1.1 Experimental Procedure

For a calorespirometric measurement soil samples must be treated before as explained in Sects. 2.1.2 and 2.1.3. Once samples are equilibrated at the temperature of the measurement and stabilized after being brought to 60% of WHC, they can be introduced inside the calorimetric ampoules (4 ml) or vessels (20 ml).

In general, an aliquot of the soil sample stabilized is introduced in one calorimetric ampoule and a second aliquot of the same soil sample will be introduced in another calorimetric ampoule carrying the vial with the NaOH (Fig. 3). Both aliquots must have the same size and be introduced simultaneously inside the calorimeter in their respective ampoules.

The number of soil aliquots for calorespirometric measurements may depend on the number of calorimetric channels available and also on the reproducibility of results. The experiment must be designed accordingly. As an example, if the goal is to compare

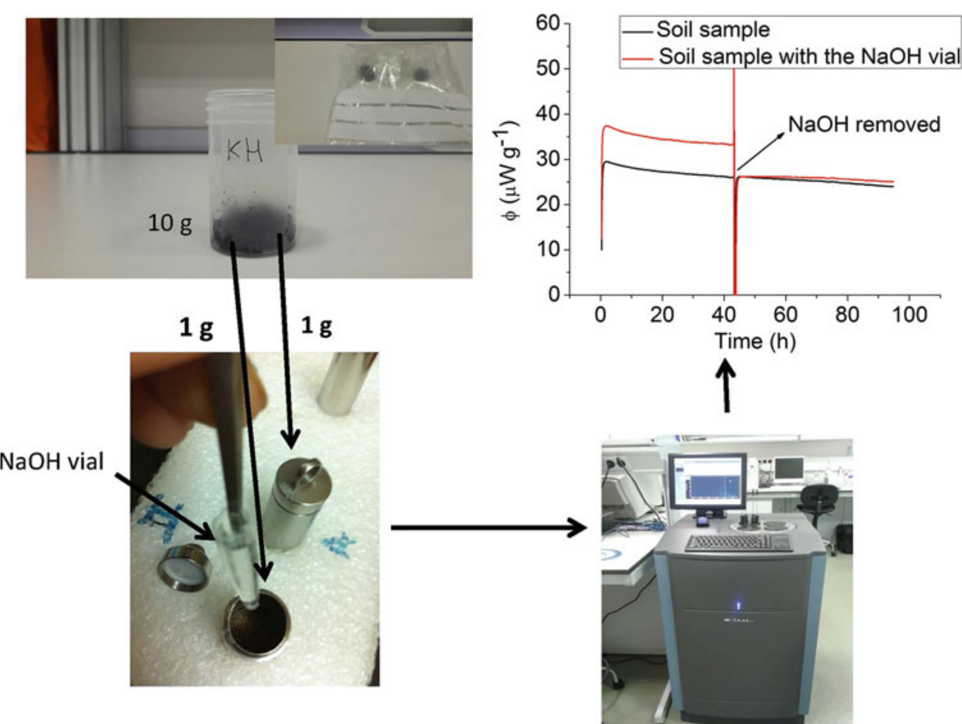

Fig. 3 Summary of the calorespirometric procedure to measure *Rq*, R_{CO_2} and CR of soil basal metabolism

the heat and CO_2 rates among different soil types and we have six calorimetric channels, six aliquots of the same soil sample can be used in each experiment; three ampoules with the soil aliquots and three ampoules with the soil aliquot and the NaOH vial. By this procedure we measure the heat and CO_2 rates with triplicates of the same soil sample providing $n = 3$ measurements for each soil. In general, one calorespirometric measurement needs two calorimetric ampoules.

The size of the vial containing the NaOH solution depends on the soil sample size and on the volume of the calorimetric vessel where the soil sample is placed. In general, for 4 ml calorimetric ampoules and 0.8–1 g of soil, a 0.2 ml vial filled with a 0.4 M solution of NaOH is used. The duration of the measurement is variable and may depend on the goal of the study. In our particular case, we have got the best results by introducing the NaOH in one of the samples at the beginning of the measurement and letting the measurement with the NaOH during 24 h. Then, the NaOH is removed and the soil sample inserted again in the calorimeter to continue the measurement with all the samples for 24 h more. By this procedure we can monitor the basal metabolism of the samples that had the NaOH to compare them with that of the samples without NaOH to check the reproducibility of the experiment.

Note that if at the end of the experiments all the plots have the same shape, no disturbance events took place in the samples with the NaOH. If that happened, it would affect the ϕ values and that would be registered in the ϕ–t plot from the sample with the NaOH. When some of the ϕ–t plots from the samples with NaOH do not follow the profile of the ϕ–t plots from the samples without NaOH along the entire measurement (48 h), the experiment must be discarded. This can happen but it is unusual.

3.1.2 Calculation of the CO_2 Rate by Calorespirometry

Figure 3 shows the plot obtained from the calorimetric ampoule with the soil and the NaOH vial and from the ampoule with the soil sample alone when measured at a metabolic steady state. It can be observed that the plot from the sample with the NaOH has the same profile as that of the soil without the NaOH but it is simply more exothermic, ϕ is higher. The reason is that inside that ampoule two different reactions are being measured: ϕ from the soil basal metabolism and ϕ from the reaction between the NaOH and the CO_2 released by the soil metabolism. The reaction between the NaOH and the CO_2 is exothermic and is written as follows:

$$CO_2(g) + 2OH^-(aq, 0.4M) \rightarrow CO_3^{2-}(aq) + H_2O\,(l) \qquad (10)$$

The enthalpy of the reaction 10 is $\Delta_{abs}H = -108.5$ kJ mol^{-1}CO_2 when using 0.4 M NaOH. If the molarity is changed, then $\Delta_{abs}H$ should be corrected accordingly [43].

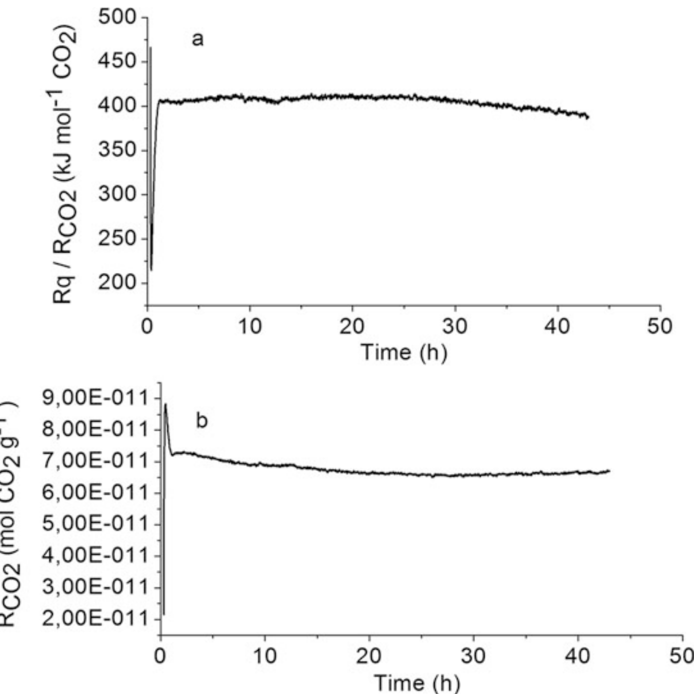

Fig. 4 Plots showing the CR (**a**) and R_{CO_2} (**b**) temporal evolution of the soil basal metabolism along the calorimetric measurement using the calculations with the tabulated ϕ data of the experiment

Calorimeters yield the ϕ data graphically and tabulated in microwatts per gram of sample. Therefore, we have two tables of ϕ data compiled for each calorespirometric measurement; one table with the ϕ data values of the basal metabolism and the reaction between the CO_2 and NaOH, and the table with the ϕ values of the basal metabolism only. With these tables, CO_2 rates (R_{CO_2}) can be calculated by two different ways to obtain the tabulated data of the R_{CO_2} values along the measurement, and an averaged value of the R_{CO_2} rate for the whole measurement time. In the first case, one determines the R_{CO_2} in mol per second, in the second case, mol of CO_2 per day or per hour. These two options involve a different procedure with the tabulated data from the calorimeter. In the first case, it is possible to show graphically the evolution of the R_{CO_2} with time during the measurement (Fig. 4) [30, 44], in the second case, one just gets a quantitative value that can be useful to compare among different soils [25, 45].

In any of these cases, it is convenient to export the tabulated data from the calorimeter to the desired data processing software.

ϕ is the microwatts from the ampoule with the soil sample and the NaOH vial and it is the sum of the ϕ from the soil basal metabolism, ϕ_{met}, and the ϕ from the reaction between the NaOH and the CO_2, ϕ_{CO_2}:

$$\phi = \phi_{met} + \phi_{CO_2} \tag{11}$$

ϕ_{met} is known from the ϕ values from the samples without the NaOH. Therefore, R_{CO_2} can be obtained by the following equation:

$$R_{CO_2} = (\phi - \phi_{met})/\Delta_{abs}H \quad (12)$$

Equation 12 can be applied to the result files with the tabulated data ϕ and ϕ_{met} to obtain a new result file with tabulated data of the R_{CO_2} in moles per second. By this way, the tabulated R_{CO_2} obtained can be plotted versus the tabulated time file representing the duration of the measurement to obtain the R_{CO_2} evolution graphically (Fig. 4). This procedure would permit detecting the immediate response of soil microbial respiration and metabolism to any external agent.

The other option is to obtain an average of the CO_2 released during the measurement. In this case

ϕ–time and ϕ_{met}–time plots must be integrated along the same interval of time, Δt. The integration is done graphically, the *y*-axis representing ϕ *and* ϕ_{met} in microwatts per gram of sample, and *x*-axis the time in seconds. This way gives the heat released in micro Joules per gram of sample. If Δt is 24 h, the heat released per day or per hour (Rq) is obtained. Rq determined from the soil sample with the NaOH is the sum of the heat rate from the soil basal metabolism (Rq_{met}) and the heat rate from the reaction between the NaOH and CO_2 (Rq_{CO_2}):

$$Rq = Rq_{met} + Rq_{CO_2} \quad (13)$$

Rq_{met} is determined by integrating the ϕ–time plots from the samples without the NaOH along the same Δt. The fact that both $\phi - t$ and ϕ_{met}–t plots are represented along exactly the same distribution of the time data, makes this approach more accurate than when done by independent experimental methods.

Applying Eq. 12, R_{CO_2} in moles per day or hour can be determined as follows:

$$R_{CO_2} = (Rq - Rq_{met})/\Delta_{abs}H \quad (14)$$

3.1.3 The Calorespirometric Ratio

The calorespirometric ratio (CR) of a certain metabolic process is the quotient between the heat rate (Rq) and the CO_2 rate (R_{CO_2}) in kilojoules per mol CO_2 (kJ $mol^{-1}CO_2$). When applied to metabolic reactions taking place without a gain in microbial carbon, the obtained values depend on the oxidation state of the substrates being metabolized [46]:

$$Rq/R_{CO_2} = (-455 \pm 15)\left[1 - \left(\gamma_{C_{sub}}/4\right)\right] \quad (15)$$

where Rq/R_{CO_2} is the CR, −455 kJ $mol^{-1}CO_2$ is Thorton's constant, and $\gamma_{C_{sub}}$ is the degree of oxidation of the substrate

being metabolized by microbial action. Equation 15 can be useful to extract information about the nature of the substrate being degraded, in particular when working with a microbial population degrading complex substrates as happens in soils.

When measured during a microbial growth reaction stimulated by the addition of an external carbon source as explained in Sect. 2, and if the enthalpy of combustion for that carbon source is known, CR allows the calculation of the metabolic carbon conversion efficiency (CUE) of the microbial growth reaction being measured:

$$\varepsilon/(1-\varepsilon) = (\mathrm{Rq}/R_{\mathrm{CO_2}} - \Delta_{\mathrm{cat}}H)/\Delta_B H \tag{16}$$

where ε is the metabolic carbon conversion efficiency of the microbial reaction, $\Delta_{\mathrm{cat}}H$ is the enthalpy of combustion for the external carbon source added, and $\Delta_B H$ is the enthalpy for the conservative reaction as shown in Eq. 5, although in this case it is not determined experimentally but by the difference between the enthalpies of combustion for the external substrate and for the microbial biomass [30, 36, 46].

Even though the application of CR to soil microbial metabolism is recent and the interpretation of CR values for soil is still under development, the literature shows good examples of these applications as well as a detailed review about CR calculations by calorimetry [43, 45, 47]. When determined by the Rq values it is obtained the averaged CR along the Δt applied to the integration of the ϕ–t plots, but it can be determined by the ϕ and $R_{\mathrm{CO_2}}$ tabulated data to show graphically the variation of the CR along the measurement (Fig. 4) being more accurate than the averaged option. The graphical CR option detects biochemical changes in the soil in real time constituting a procedure to detect immediate biochemical changes linked to any external agent.

4 Future Trends: Calorimetry and the Influence of Temperature on Soil Biodegradation

The relation between temperature and the biodegradation of the soil organic matter is one of the less explored subjects in soil science. Most of the results in the literature compile variation of the CO_2 rates of the soil microbial metabolism or different enzymatic activities along increasing temperatures ranging from about 4 °C to 35 °C. The sensitivity of the soil microbial system to increasing temperature is mainly done by the calculation of the Q10 or by exploring how the obtained rates fit with the Arrhenius equation. The role of the nature of the organic matter in soil with that sensitivity is still controversial and poorly understood and there is a complete lack of information about the role of extreme temperatures on the soil microbial metabolism, about the recuperation

of the soil after exposed at temperatures higher than 35 °C or about the reaction of soil to cooling instead of heating. The main reason is that the methodological options to develop those experiments are scarce.

New calorimeters may have an important role in the development of that pending subject since some of them are designed to allow temperature to change during the measurement. This option has been introduced now by new calorimeters as TAM III and TAM IV (TA Instruments). Recent papers showed the immediate response of soil basal metabolism to changing temperature from 18 °C to 35 °C using this device and that calorimetry allows the calculation of the Q10 and the application of the Arrhenius equation by the heat rate of the soil microbial metabolism [48, 49]. The heat rate used was the direct measurement of the ϕ data obtained at each temperature but that is possible if the thermal power is almost constant at a certain temperature. The most recent results, still unpublished, may show that not all the soils are capable of keeping a constant metabolic rate as temperature increases. In this case, it is more convenient the use of the averaged heat rate in Joules per day or hour that involves the integration of the $\phi - time$ plots at each temperature as explained along this chapter.

Our last applications to soils with different organic matter properties have shown different responses to increasing temperature, suggesting that the concept of sensitivity of the soil organic matter to temperature may depend on features beyond the Q10 values or the Arrhenius behavior. Figure 5 shows the $\phi - t$ plots

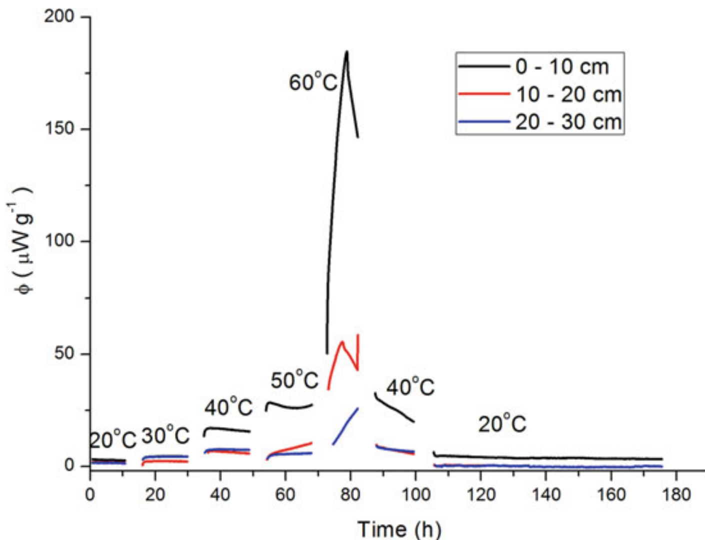

Fig. 5 ϕ temporal evolution at different temperatures of a soil sample collected from different depths. The superficial soil (0–10 cm) resists the scan to 60 °C fitting the Arrhenius model from 20 °C to 60 °C. As depth increases the range of temperatures at which the ϕ values fit the Arrhenius model decrease being from 20 °C to 40 °C in the deepest sample. The middle and deepest sample did not recover the initial microbial activity at 20 °C after the temperature scan

obtained under a scan of temperature from 20 °C to 60 °C followed by the cooling from 60 °C to 40 °C and to 20 °C inserting isothermal phases of about 15 h. Soil samples for these measurements were collected at different depths differing in the organic matter properties. The direct observation of the $\phi - t$ curves (Fig. 5) clearly shows how soils vary in their resistance to the extreme temperatures. The analysis of the data from Fig. 5 showed that the biodegradation rates fitted the Arrhenius model only at a certain interval of temperatures depending on the soil sample.

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Chapter 8

Improved Model for Biodegradability of Organic Compounds: The Correlation Contributions of Rings

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Abstract

The CORAL software was utilized to build up predictive model for biodegradability of organic compounds. The model was calculated with correlation weights of attributes of simplified molecular input-line entry system (SMILES). The previous model of the endpoint calculated with the CORAL software has been based on the attributes extracted from SMILES, which reflect the presence of various atoms and covalent bonds. In this work, the attributes of different rings (size, presence of heteroatoms) are involved in the modeling process. The comparison of these models with models where rings were not taken into account has shown significant improvement of the statistical quality of the biodegradation prediction.

Key words QSAR, Biodegradability, Monte Carlo method, CORAL software

1 Biodegradability

Biodegradability is an important ecological indicator of a substance since the value of biodegradability gives possibility of predicting and describing the fate of the substance as a pollutant of an ecologic system (air, water, or soil). The environmental fate of transformation products from organic pollutants such as drugs has become a new research area of increasing interest [1]. Assessing the environmental fate (biodegradability) of pesticides also is a very important task for agricultural industry and medicine [2]. In the European Chemicals Agency (ECHA) guidelines, biodegradation is defined as the biologically mediated degradation or transformation of chemicals carried out by microorganisms. Most of the models generate qualitative predictions (i.e., ready vs. non-ready biodegradability) [3]. The most common procedures to assess biodegradability are the OECD 301F and 301C tests [4].

Despite prevalence of the above-mentioned qualitative models (ready—non ready biodegradability) [5–7], there are attempts to

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build up the quantitative models, i.e., quantitative structure—activity relationships (QSAR)Quantitative structure—activity relationships (QSAR) oriented to predict the biodegradability numerically [8–17]. Unfortunately, the above-mentioned works are very unstandardized. The comparison of the statistical quality of these works sometimes is impossible owing to the eclectic variations of substances involved in the datasets as well as owing to specificity of criteria selected by different authors to assess the corresponding predictive potentials.

The CORAL software (http://www.insilico.eu/coral) can be used to define a standardized model for different endpoints. Below, the application of the software for the case of building up QSAR models for biodegradability is given.

2 The CORAL Software

The CORAL software has been developed [18–20] as a tool for building up quantitative structure—property/activity relationships (QSPRs/QSARs). The general scheme of application of the software is the following:

1. Preparation of the total set of available data on endpoint in the form: "ID-SMILES-Endpoint" for each substance;
2. Distribution of the available data into training set, invisible training set, calibration set, and external validation set;
3. Definition of mathematical equation for so-called optimal descriptor;
4. Building up a model; and
5. Checking up the predictive potential of the model.
   Thus, the CORAL model in a generalized form is the following:

$$\text{Optimal Descriptor} = \text{F (SMILES)} \quad (1)$$

$$\text{Endpoint} = \text{C0} + \text{C1} \times \text{Optimal Descriptor} \quad (2)$$

The definition of the mathematical form for an optimal descriptor aimed to build up a QSAR model is a select of a group of basic molecular features, which can be extracted from SMILES. An user can select features extracted solely from SMILES or solely from molecular graph as well as the user can define hybrid descriptor calculated with attributes of SMILES together with invariants of a molecular graph (Fig. 1). The software can involve three types of the molecular graphs [21, 22]: (1) hydrogen suppressed graph (HSG); (2) hydrogen filled graph (HFG); and (3) graph of atomic orbitals (GAO).

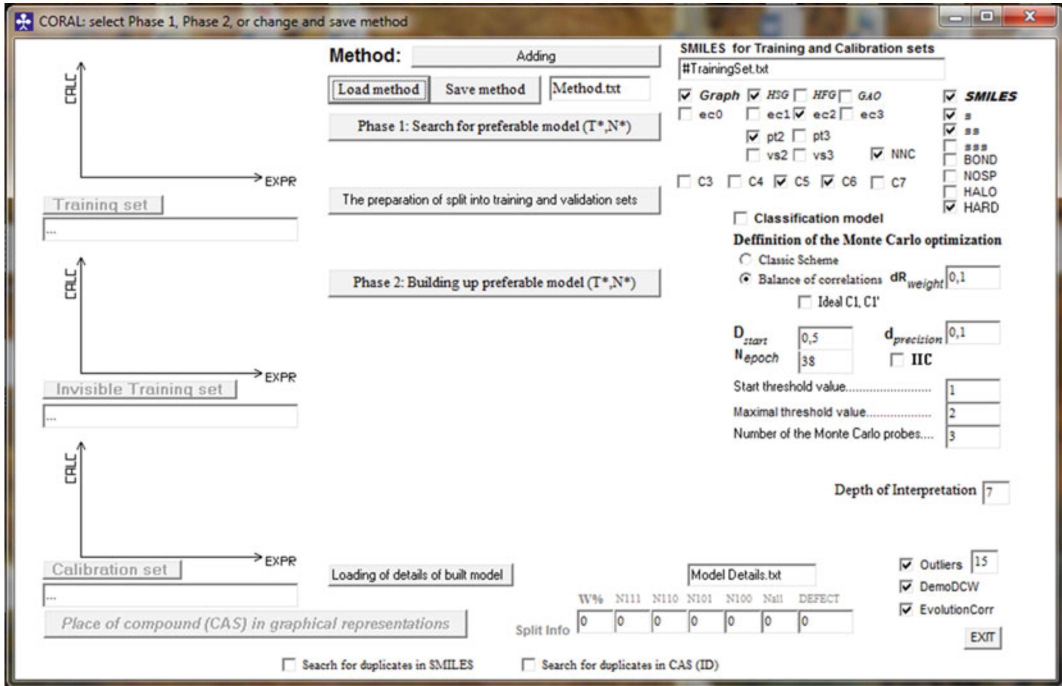

Fig. 1 Interface for the CORAL software

The list of available SMILES attributes is the following (Fig. 1):

1. SMILES atoms (denoted s), which are one symbol or two symbols which cannot be examined separately (e.g., "Cl," "Br," etc.);
2. Combining of two SMILES atoms (denoted ss);
3. Combining of three SMILES atoms (denoted sss);
4. Global SMILES attributes BOND, NOSP, HALO, and HARD are represented in Table 1. The BOND is a mathematical function of presence/absence of different kinds of chemical bonds (i.e., double, denoted "="; triple denoted "#"; and stereo chemical denoted "@"); The NOSP is a mathematical function of presence/absence nitrogen (N), oxygen (O), sulfur (S), and phosphorus (P); the HALO is a mathematical function of presence/absence of halogens (i.e., fluorine, chlorine, bromine, and iodine). Finally, the HARD is association of BOND, NOSP, and HALO in united structural code (Table 1).

The list of available graph invariants is the following (Fig. 1):

1. Vertex degree (i.e., the number of vertices that are neighbors for the given vertex), denoted "ec0";
2. The extended connectivity of the first order is denoted as "ec1";
3. The extended connectivity of the second order is denoted as "ec2";
4. The extended connectivity of the third order is denoted as "ec3";

Table 1
Examples of representation of SMILES attributes by means of 12 symbols [SMILES = "NC(SCCF) = N"]

ID	Comment	1	2	3	4	5	6	7	8	9	10	11	12
1	Representation of S_k	**N** **C** **(*** **S** **C** **C** **F** **(** **=** **N**	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
2	Representation of SS_k	**N** **C** **S** **S** **C** **F** **F** **=** **N**	. . . .	. . . .	. . . .	**C** **(** **(** **C** **C** **C** **(** **(** **=**	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
3	Definition of BOND	**B**	**O**	**N**	**D**	= **1**	# **0**	@ **0**	**0**	**0**	**0**	**0**	**0**
4	Definition of NOSP	**N**	**O**	**S**	**P**	N **1**	O **0**	S **1**	P **0**	**0**	**0**	**0**	**0**
5	Definition of HALO	**H**	**A**	**L**	**O**	F **1**	Cl **0**	Br **0**	I **0**	**0**	**0**	**0**	**0**
6	Definition of HARD	**$**	= **1**	# **0**	@ **0**	N **1**	O **0**	S **1**	P **0**	F **1**	Cl **0**	Br **0**	I **0**

5. The number of paths of length 2, which are started from a given vertex is denoted as "pt2";
6. The number of paths of length 3, which are started from a given vertex is denoted as "pt3";
7. Valence shell of second order is denoted as "vs2";
8. Valence shell of third order is denoted as "vs3";
9. Nearest neighbors code is denoted as "NNC";
10. The descriptors, which are reflected presence/absence of rings, are denoted as C3-C7 for rings, which include from three members till seven members, respectively [23]. These features are reflecting presence/absence in the rings of hetero atoms and aromaticity [23].

3 Sources of Data

Two data sets on the biodegradability are studied. The dataset 1 is a set of organic substances taken in work [3]. The dataset 2 is a set of organic substances taken in work [4]. Three random and considerable different splits into the training (≈40%), invisible training (≈40%), calibration (≈10%), and validation (≈10%) sets have been prepared for these two datasets. Table 2 contains data on percentage of identity for these splits.

Table 2
Percentage of identity for examined splits into the training set, invisible training set, calibration and validation sets for dataset 1 ($n = 445$) and dataset 2 ($n = 59$)

Split	Set	445-1	445-2	445-3
445-1	Training	100*	44.7	43.1
	Invisible training	100	43.4	42.7
	Calibration	100	16.5	16.5
	Validation	100	18.3	27.3
445-2	Training		100	39.2
	Invisible training		100	35.8
	Calibration		100	18.0
	Validation		100	17.8
445-3	Training			100
	Invisible training			100
	Calibration			100
	Validation			100
		59-1	59-2	59-3
59-1	Training	100	30.8	43.9
	Invisible training	100	29.4	29.4
	Calibration	100	18.2	38.1
	Validation	100	8.7	27.3
59-2	Training		100	35.0
	Invisible training		100	38.9
	Calibration		100	9.5
	Validation		100	19.0
59-3	Training			100
	Invisible training			100
	Calibration			100
	Validation			100

$\text{Identity}(\%) = \frac{N_{i,j}}{0.5^*(N_i+N_j)} \times 100$

$N_{i,j}$ is the number of substances which are distributed into the same set for both ith split and jth split (set = training, invisible training, calibration, and validation)

N_i is the number of substances which are distributed into the set for ith split

N_j is the number of substances which are distributed into the set for jth split

4 Building Up QSAR Models

The hybrid optimal descriptor [24] selected for the QSAR has been defined as the following:

$$^{\text{hybrid}}\text{DCW}(T, N) = {}^{\text{SMILES}}\text{DCW}(T, N) + {}^{\text{HSG}}\text{DCW}(T, N) \quad (3)$$

where

$$^{\text{SMILES}}\text{DCW}(T, N) = \text{CW}(\text{HARD}) + \sum \text{CW}(S_k) + \sum \text{CW}(SS_k) \quad (4)$$

$$^{\text{HSG}}\text{DCW}(T, N) = \text{CW}(C5) + \text{CW}(C6) + \sum \text{CW}(\text{ec1}_k) + \sum \text{CW}(pt2_k) + \sum \text{CW}(\text{NNC}_k) \quad (5)$$

The CW(x) is a correlation weight for a SMILES attribute or an invariant of HSG. The numerical data on the correlation weights are optimized by the Monte Carlo method in order to obtain a maximal value of the target function (TF):

$$\text{TF} = R + R' - |R - R'| \times dR \quad (6)$$

The R and R' are correlation coefficients for correlation between biodegradability and descriptor calculated with Eq. 3 for training set and invisible training set, respectively.

The T is threshold to define rare molecular features. The N is number of epochs of the optimization [23, 24]. Threshold is an integer (e.g., 1, 2, 3,. . .) which used to separate molecular features (SMILES attributes and/or invariants of graph) into two classes (1) rare; and (2) active. The correlation weights of rare features are fixed equal to zero, i.e., these features are not involved into the modeling process.

Basic hypothesis is the $T = T^*$ and $N = N^*$ which give the best statistics for calibration set should be used to build up the model:

$$\text{Biodegradability} = \text{C0} + \text{C1} \times \text{DCW}(T^*, N^*) \quad (7)$$

Final checking of the predictive potential of a model which is calculated with Eq. 7 should be carried out with chemicals of the external validation set.

5 Assessment of QSAR Models for Biodegradability

Table 3 contains the statistical characteristics of the QSAR for biodegradability. One can see the statistical quality of these models is quite good for all splits and both datasets. These models are calculated with the following equations:

Table 3
The statistical characteristics of QSAR for biodegradability

Dataset	Split	Set	n	R^2	q^2	RMSE
1	1 Model with C5&C6	Training	164	0.6592	0.6508	0.227
		Invisible training	163	0.6605	0.6525	0.242
		Calibration	59	0.7631		0.204
		Validation	59	0.7771		0.200
	1 Model without C5&C6	Training	164	0.6427	0.6318	0.232
		Invisible training	163	0.6377	0.6286	0.247
		Calibration	59	0.7527		0.204
		Validation	59	0.7237		0.225
	2 Model with C5&C6	Training	167	0.7149	0.7088	0.211
		Invisible training	178	0.6556	0.6499	0.231
		Calibration	50	0.8398		0.176
		Validation	50	0.8615		0.150
	2 Model without C5&C6	Training	167	0.7005	0.6945	0.216
		Invisible training	178	0.6265	0.6200	0.190
		Calibration	50	0.8471		0.248
		Validation	50	0.8548		0.179
	3 Model with C5&C6	Training	170	0.6074	0.6003	0.249
		Invisible training	174	0.6238	0.6167	0.231
		Calibration	50	0.8467		0.193
		Validation	51	0.8082		0.224
	3 Model without C5&C6	Training	170	0.5768	0.5686	0.258
		Invisible training	174	0.5963	0.5878	0.240
		Calibration	50	0.8117		0.220
		Validation	51	0.7894		0.213
2	1 Model with C5&C6	Training	20	0.8365	0.7789	0.059
		Invisible training	16	0.7864	0.6396	0.069
		Calibration	11	0.6279		0.046
		Validation	12	0.6769		0.066
	2 Model with C5&C6	Training	19	0.8713	0.8451	0.035
		Invisible training	18	0.9291		0.130
		Calibration	11	0.8998		0.049
		Validation	11	0.6124		0.057
	3 Model with C5&C6	Training	21	0.7453	0.6202	0.073
		Invisible training	18	0.7450	0.6102	0.070
		Calibration	10	0.7416		0.086
		Validation	10	0.7404		0.057

Dataset 1

$$\text{Split 1: Biodegradability} = -0.0705050 + 0.0345563^{*}\text{DCW}(1, 38) \quad (8)$$

$$\text{Split 2: Biodegradability} = -0.0369133 + 0.0476929^{*}\text{DCW}(1, 40) \quad (9)$$

$$\text{Split 3 : Biodegradability} = 0.1361130 + 0.0299055^{*}\text{DCW}(1, 22) \tag{10}$$

Dataset 2

$$\text{Split 1: Biodegradability} = -0.1172974 + 0.0095387^{*}\text{DCW}(1, 23) \tag{11}$$

$$\text{Split 2: Biodegradability} = 0.0730784 + 0.0052729^{*}\text{DCW}(1, 27) \tag{12}$$

$$\text{Split 3: Biodegradability} = 0.0261062 + 0.0061330^{*}\text{DCW}(1, 25) \tag{13}$$

Table 4 contains experimental [3] and calculated values of the biodegradability for dataset 1. Table 5 contains experimental [4] and calculated values of biodegradability for dataset 2.

6 Mechanistic Interpretation

Having numerical data on the correlation weights of molecular features extracted from SMILES and graph for several runs of the optimization procedure, one can obtain features of three categories:

1. Features with positive correlation weights in all runs, which should be considered promoters of increase for biodegradability;
2. Features with negative correlation weights in all runs, which should be considered promoters of decrease for biodegradability; and
3. Features that have positive and negative correlation weights in different runs: the influence of such features for biodegradability is unclear.

The comparison of lists of promoters of increase/decrease for biodegradability of different splits gives possibility of estimating significance of different molecular features for biodegradability from the probabilistic point of view. The frequency of a feature in the training, invisible training, and calibration sets should be taken into account for the correctness of the comparisons. This comparison can be especially interesting for different datasets. Table 6 contains comparison of features, which are promoters of increase or decrease for biodegradability for first splits from dataset 1 and dataset 2. There are 16 of molecular features with significant prevalence, which are acting in an equivalent manner for both the cases, i.e., they are promoters of increase of biodegradability for both the

Table 4
Experimental and predicted values of biodegradability for three random splits into the training (T), invisible training (I), calibration (C), and validation (V) sets (dataset 1, $n = 445$)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
T	I	V	56-81-5	OCC(O)CO	0.6300	0.8716	0.8078	0.8011
I	T	C	57-57-8	O=C1CCO1	0.7850	0.9949	1.0442	0.9289
V	V	T	60-12-8	OCCc1ccccc1	0.8700	0.7921	0.6519	0.7335
C	V	C	65-85-0	O=C(O)c1ccccc1	0.8500	0.6260	0.6219	0.6026
V	I	T	66-72-8	Oc1c(C=O)c(cnc1C)CO	0.7700	0.7320	0.7672	0.5834
I	I	I	67-52-7	O=C1CC(=O)NC(=O)N1	0.7600	0.5688	0.4414	0.6131
I	C	V	78-81-9	CC(C)CN	0.8700	0.7281	0.7226	0.5511
C	V	T	78-84-2	CC(C)C=O	0.8100	0.8692	1.0081	0.8407
V	I	T	79-10-7	C=CC(=O)O	0.6800	0.8715	0.8296	0.7675
V	T	T	79-11-8	ClCC(=O)O	0.6500	0.6909	0.7428	0.8037
I	I	T	79-43-6	ClC(Cl)C(=O)O	0.9700	0.5829	0.5817	0.5577
C	I	T	85-44-9	O=C1OC(=O)c2ccccc12	0.8520	1.3157	1.0331	0.9913
V	I	T	87-20-7	Oc1ccccc1C(=O)OCCC(C)C	0.8340	0.9996	0.9613	0.9298
I	T	I	88-06-2	Clc1cc(Cl)cc(Cl)c1O	0.8250	0.2973	0.1671	0.1237
T	T	I	91-16-7	COc1ccccc1OC	0.9600	0.6488	0.6491	0.5581
I	I	I	91-64-5	O=C1C=Cc2ccccc2O1	1.0000	0.7687	1.0130	1.0394
T	T	T	92-52-4	c1cc(ccc1)c2ccccc2	0.6600	0.4260	0.3637	0.2911
V	T	T	93-10-7	O=C(O)c1ccc2ccccc2n1	0.8100	0.6541	0.8475	0.8474
T	I	T	94-36-0	O=C(OOC(=O)c1ccccc1)c2ccccc2	0.8400	0.7617	0.9016	0.7527
I	I	I	96-09-3	c1ccccc1C2CO2	0.8100	0.5294	0.6197	0.5135

(continued)

Table 4
(continued)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
T	I	C	96-24-2	OCC(O)CCl	0.6800	0.5024	0.5936	0.3812
T	C	I	96-48-0	O=C1CCCO1	0.7600	0.8868	1.0075	0.9327
C	I	T	96-49-1	O=C1OCCO1	0.6410	0.3901	0.4713	0.5565
T	T	T	96-54-8	Cn1cccc1	0.7800	0.7704	0.7752	0.7748
I	C	V	98--0	OCc1ccco1	0.7700	0.7516	0.8551	0.5674
I	T	T	98-55-5	CC1=CCC(CC1)C(O)(C)C	0.8460	0.5993	0.6616	0.4216
T	I	V	98-86-2	CC(=O)c1ccccc1	0.6470	0.6265	0.7108	0.6089
T	T	I	98-87-3	ClC(Cl)c1ccccc1	0.9050	0.8343	0.7649	0.4934
I	T	T	99-87-6	Cc1ccc(cc1)C(C)C	0.8900	0.5767	0.5568	0.4785
T	T	I	1-42-5	C=Cc1ccccc1	1.0000	0.5217	0.4799	0.4367
T	T	T	1-44-7	ClCc1ccccc1	0.7100	0.6083	0.4883	0.3791
I	I	I	1-52-7	O=Cc1ccccc1	0.6600	0.8480	0.8993	0.8195
T	I	T	101-83-7	C1CCCCC1NC2CCCCC2	0.7690	0.2897	0.1978	0.1515
C	V	I	103-73-1	CCOc1ccccc1	0.6300	0.7753	0.7357	0.7579
T	T	T	107-18-6	C=CCO	0.8600	0.9298	0.9019	0.7964
T	I	T	107-22-2	O=CC=O	0.6500	0.8757	1.0324	0.9513
C	I	C	108-05-4	CC(=O)OC=C	0.9000	0.8697	0.8658	0.8838
I	V	I	108-10-1	CC(C)CC(C)=O	0.8400	0.8312	0.7936	0.7610
T	T	I	108-88-3	Cc1ccccc1	1.0000	0.8119	0.7707	0.6871
V	C	V	109-86-4	OCCOC	0.8350	0.9496	1.0083	0.9213

T	T	I	109-99-9	C1CCCO1	1.0000	0.6339	0.7187	0.6283
V	I	C	110-58-7	CCCCCN	0.7400	0.7766	0.7100	0.6677
C	T	V	110-63-4	OCCCCO	0.8500	0.8740	0.8260	0.8745
I	V	I	110-80-5	CCOCCO	0.7300	0.6859	0.7075	0.8090
V	T	V	112-70-9	CCCCCCCCCCCCCO	0.8840	0.8246	0.7722	0.8129
T	T	I	112-84-5	NC(=O)CCCCCCCCCCC\C=C\CCCCCCCC	0.8800	0.6584	0.7589	0.5774
I	T	T	119-68-6	CNc1ccccc1C(=O)O	0.8500	0.4404	0.5214	0.5448
T	I	T	120-57-0	O=Cc1ccc2OCOc2c1	0.8800	1.0315	0.9557	1.0904
T	I	T	120-61-6	COC(=O)c1ccc(cc1)C(=O)OC	0.8400	0.8451	0.9295	0.7884
C	T	V	120-80-9	Oc1ccccc1O	0.8300	0.9800	0.9532	0.8420
C	C	V	121-91-5	OC(=O)c1cccc(c1)C(=O)O	0.7770	0.8713	0.7712	0.7096
T	I	C	123-31-9	Oc1ccc(O)cc1	0.7000	0.6576	0.5638	0.5575
T	T	T	123-35-3	C/C(C)=C\CCC(=C)C=C	0.8700	0.5216	0.5122	0.4444
T	I	I	123-42-2	CC(=O)CC(C)(C)O	0.9000	0.7839	0.5282	0.5331
I	I	C	123-72-8	CCCC=O	1.0000	0.8609	0.9963	0.9143
C	I	T	124-04-9	OC(=O)CCCCC(=O)O	0.7900	0.9739	0.8217	0.8970
T	T	T	127-19-5	CN(C)C(C)=O	0.8300	0.6883	0.6265	0.6045
T	I	I	135-19-3	Oc1ccc2ccccc2c1	0.6800	0.3401	0.3945	0.4698
V	T	V	138-86-3	CC1=CCC(CC1)C(C)=C	0.6950	0.3983	0.4888	0.4183
T	T	C	140-29-4	N#CCc1ccccc1	0.7700	0.6147	0.7627	0.5282
V	C	T	141-17-3	CCCCOCCOCCOC(=O)CCCCC(=O)OCCOCCOCCCC	0.8600	0.7965	0.9671	0.8608
T	I	I	141-32-2	C=CC(=O)OCCCC	0.6100	0.8244	0.8081	0.7871
T	I	T	142-08-5	Oc1ccccn1	0.6800	0.4881	0.4556	0.4362

(continued)

Table 4 (continued)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
T	I	T	482-05-3	OC(=O)c2ccccc2c1ccccc1C(=O)O	1.0000	0.5870	0.5154	0.5191
T	T	C	495-69-2	O=C(NCC(=O)O)c1ccccc1	0.8100	0.7413	0.6926	0.5114
T	T	I	499-83-2	OC(=O)c1cccc(n1)C(=O)O	0.7900	0.7985	0.6189	0.6705
T	T	I	517-23-7	CC(=O)C1CCOC1=O	0.7200	0.9170	1.1952	1.0752
C	I	V	520-45-6	O=C(C)C1C(=O)C=C(C)OC1=O	0.8400	0.7498	0.8129	0.7447
T	T	V	520-45-8	OC(=C)C=1C(=O)C=C(C)OC=1O	0.8400	0.7470	0.6054	0.4828
C	V	I	520-45-9	O=C(C)C1=C(O)C=C(C)OC1=O	0.8400	0.7295	0.7987	0.6677
V	C	I	520-45-10	O/C(C)=C1/C(=O)C=C(C)OC1=O	0.8400	0.9962	1.0275	0.8480
T	I	T	536-60-7	OCc1ccc(cc1)C(C)C	0.8460	0.5466	0.6013	0.6055
T	T	T	536-66-3	CC(C)c1ccc(cc1)C(=O)O	0.8850	0.5487	0.6158	0.6107
I	T	T	552-16-9	O=[N+]([O-])c1ccccc1C(=O)O	1.0000	0.7756	0.7379	0.7632
T	I	I	588-46-5	CC(=O)NCc1ccccc1	0.7700	0.5376	0.5391	0.3284
V	C	C	591-60-6	O=C(CC(C)=O)OCCCC	0.8400	0.8552	0.8464	0.8340
I	V	C	626-86-8	O=C(CCCCC(=O)O)OCC	0.8400	1.0470	0.9890	1.0056
I	T	T	645-62-5	CCC\C=C(/CC)C=O	0.8940	0.5153	0.5387	0.5970
T	C	C	821-38-5	O=C(O)CCCCCCCCCCCCC(=O)O	0.8950	0.8179	0.7114	0.7842
C	T	C	868-77-9	C=C(C)C(=O)OCCO	0.9600	0.7895	0.8650	0.7512
V	V	V	869-29-4	C=CC(OC(=O)C)OC(=O)C	0.8000	0.8032	0.7832	0.8124
V	C	C	1459-93-4	COC(=O)c1cccc(c1)C(=O)OC	0.9800	0.9922	0.9865	0.7023
V	C	V	2415-85-2	O=C(Nc1ccc(C)cc1)CC(C)=O	0.7360	0.5174	0.6369	0.5090

C	T	T	2432-87-3	CCCCCCCCOC(=O)CCCCCCCCC(=O)OCCCCCCCC	0.7820	1.0824	1.0956	1.0499
V	C	V	2517-43-3	CC(CCO)OC	0.8380	0.5647	0.5881	0.6428
C	I	T	4435-53-4	CC(CCOC(=O)C)OC	0.6350	0.8091	0.8560	0.8317
T	T	T	4719-04-4	OCCN1CN(CN(C1)CCO)CCO	0.9200	0.8913	0.9273	0.9260
C	I	T	13562-76-0	CC(CC)OC(=O)CC(C)=O	0.8500	0.8298	0.7454	0.7929
C	I	I	62-23-7	O=[N+]([O-])c1ccc(cc1)C(=O)O	0.6200	0.4674	0.5804	0.5077
I	V	V	69-72-7	OC(=O)c1ccccc1O	0.8810	0.7750	0.6757	0.6869
T	I	T	75-07-0	CC=O	0.8000	0.7892	0.4959	0.8033
I	I	T	75-50-3	CN(C)C	0.9200	0.7976	0.9366	0.9290
C	I	V	78-83-1	CC(C)CO	0.9000	0.8672	0.9249	0.7864
C	C	V	78-92-2	CC(O)CC	0.7300	0.7332	0.7642	0.6500
V	I	V	80-62-6	C=C(C)C(=O)OC	0.9400	0.7418	0.7967	0.6655
I	T	T	85-68-7	O=C(OCc1ccccc1)c2ccccc2C(=O)OCCCC	0.8100	0.6074	0.6301	0.6120
I	I	T	90-15-3	Oc2cccc1ccccc12	0.9600	0.5385	0.4064	0.4156
I	C	C	90-43-7	Oc2ccccc2c1ccccc1	0.6650	0.5897	0.5449	0.5835
C	T	C	98-01-1	O=Cc1ccco1	0.9350	0.7536	0.9383	0.6217
C	I	I	98-11-3	O=S(O)(=O)c1ccccc1	0.8700	0.9213	0.8965	0.8480
I	V	C	1-21-0	OC(=O)c1ccc(cc1)C(=O)O	0.7500	0.6763	0.6131	0.7242
V	C	C	1-46-9	NCc1ccccc1	0.7650	0.7070	0.6138	0.5299
I	C	I	1-47-0	N#Cc1ccccc1	0.6340	0.6293	0.8377	0.5132
I	I	C	1-51-6	OCc1ccccc1	0.9400	0.8460	0.8161	0.7651
I	T	I	103-84-4	O=C(C)Nc1ccccc1	0.6870	0.6871	0.6827	0.6424
I	I	I	104-94-9	Nc1ccc(OC)cc1	0.6530	0.1785	0.1509	0.1812

(continued)

Table 4 (continued)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
C	T	V	105-60-2	O=C1CCCCCN1	0.8200	0.6082	0.6196	0.5785
V	V	C	107-21-1	OCCO	0.8950	0.9429	0.9464	0.8744
V	I	T	108-01-0	CN(C)CCO	0.6050	0.5754	0.5883	0.5846
C	T	T	108-46-3	Oc1cccc(O)c1	0.6670	0.5865	0.5482	0.5147
C	C	T	108-94-1	O=C1CCCCC1	0.8700	0.6705	0.6962	0.7613
V	V	C	108-95-2	Oc1ccccc1	0.8500	0.8722	0.8422	0.8810
I	I	T	109--2	Oc1cccnc1	0.9300	0.4901	0.5362	0.5015
C	V	V	109-73-9	CCCCN	0.8500	0.7591	0.7998	0.6713
T	T	I	110-89-4	C1CCCCN1	0.6690	0.2561	0.2591	0.2153
I	C	C	111-15-9	CC(=O)OCCOCC	0.8690	0.8805	1.0199	0.9529
T	V	C	111-76-2	CCCCOCCO	0.9600	0.7920	0.7782	0.7982
I	I	C	50-31-7	Clc1c(C(=O)O)c(Cl)ccc1Cl	0.0100	0.1349	0.0606	0.1052
T	C	T	51-28-5	O=[N+]([O-])c1cc(ccc1O)[N+]([O-])=O	0.0000	−0.0107	−0.0192	0.0722
T	I	C	56-35-9	CCCC[Sn](CCCC)(CCCC)O[Sn](CCCC)(CCCC)CCCC	0.0200	0.0428	−0.0224	−0.1744
I	I	I	57-15-8	ClC(Cl)(Cl)C(C)(C)O	0.1300	−0.2355	0.0506	−0.0613
I	I	I	58-90-2	Clc1cc(Cl)c(Cl)c(Cl)c1O	0.0700	0.3729	0.3160	0.2362
I	T	T	60-29-7	CCOCC	0.4000	0.7338	0.7289	0.7554
I	T	T	60-51-5	COP(=S)(OC)SCC(=O)NC	0.0000	0.1168	0.0258	−0.0257
I	I	C	64-67-5	O=S(=O)(OCC)OCC	0.8900	0.8432	0.9088	0.4093
I	T	I	66-81-9	O=C2CC(CC(O)C1CC(C)CC(C)C1=O)CC(=O)N2	0.0100	0.1137	0.0067	0.1007

I	I	T	68-11-1	O=C(O)CS	1.0000	0.9536	1.0332	1.0141
T	T	V	70-30-4	Clc2c(Cc1c(O)c(Cl)cc(Cl)c1Cl)c(O)c(Cl)cc2Cl	0.0000	−0.0411	−0.1828	−0.5785
C	T	I	74-83-9	BrC	0.1600	0.1346	0.1603	0.1173
T	T	T	74-87-3	CCl	0.5000	0.4954	0.5020	0.4955
I	V	T	74-96-4	BrCC	0.2900	0.4650	0.1753	0.3471
T	I	I	74-97-5	BrCCl	0.6000	0.4785	0.3690	0.5549
T	V	C	75--3	CCCl	0.0100	0.0311	0.0194	0.0991
I	I	I	75-09-2	ClCCl	0.1550	0.3079	0.2992	0.3198
T	I	T	75-25-2	BrC(Br)Br	0.0000	0.0142	0.0816	−0.0066
T	I	T	75-26-3	BrC(C)C	0.8100	0.5065	0.5735	0.4168
I	T	I	75-35-4	C=C(Cl)Cl	0.0000	0.0407	0.0045	−0.0130
I	I	I	75-45-6	ClC(F)F	0.0000	0.1157	0.0437	0.1961
T	I	V	75-56-9	CC1CO1	0.9550	0.8287	0.9076	0.7740
I	T	I	75-65-0	CC(C)(C)O	0.0250	0.4744	0.5135	0.3940
I	T	T	75-86-5	N#CC(C)(C)O	0.9500	0.4371	0.9469	0.7350
V	I	I	75-87-6	ClC(Cl)(Cl)C=O	0.0700	0.2659	0.1143	0.2995
I	T	I	76-03-9	ClC(Cl)(Cl)C(=O)O	0.2300	0.3287	0.4077	0.3274
I	I	I	76-12-0	ClC(Cl)(F)C(Cl)(Cl)F	0.1800	−0.3306	−0.0710	0.1289
V	I	T	76-13-1	ClC(F)(F)C(Cl)(Cl)F	0.0250	−0.0478	0.1588	0.4540
T	T	I	76-44-8	ClC2=C(Cl)C3(Cl)C1C=CC(Cl)C1C2(Cl)C3(Cl)Cl	0.0000	−0.6174	−0.0089	−0.0245
T	I	I	76-83-5	ClC(c1ccccc1)(c2ccccc2)c3ccccc3	0.0000	0.0114	−0.0383	−0.0532
T	I	T	76-87-9	O[Sn](c1ccccc1)(c2ccccc2)c3ccccc3	0.0000	0.0354	−0.0199	0.0433
T	T	I	76-93-7	OC(C(=O)O)(c1ccccc1)c2ccccc2	0.0000	−0.0255	−0.0103	0.2657

(continued)

Table 4 (continued)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
I	T	T	77-68-9	CC(C)(COC(=O)C(C)C)C(O)C(C)C	0.9600	0.8274	0.8167	0.7676
T	I	I	77-90-7	CCCCOC(=O)CC(CC(=O)OCCCC)(OC(C)=O)C(=O)OCCCC	0.8200	0.7665	0.8508	0.7638
C	T	I	78-30-8	Cc3ccccc3OP(=O)(Oc1ccccc1C)Oc2ccccc2C	0.6570	0.9638	0.6545	0.6990
I	T	I	78-40-0	CCOP(=O)(OCC)OCC	0.0000	0.2438	0.1887	0.1388
C	T	T	78-42-2	CCCCC(COP(=O)(OCC(CC)CCCC)OCC(CC)CCCC)CC	0.0000	−0.4043	−0.1441	−0.0372
T	C	I	78-51-3	CCCCOCCOP(=O)(OCCOCCCC)OCCOCCCC	0.0000	0.0622	0.1401	0.0168
I	I	T	78-79-5	C=C(C)C=C	0.0200	0.4561	0.4667	0.3663
T	T	I	79--5	ClCC(Cl)Cl	0.0500	0.0603	−0.0382	0.1818
I	I	I	79-34-5	ClC(Cl)C(Cl)Cl	0.0000	0.1641	0.0568	0.0400
I	I	T	79-92-5	CC2(C)C(=C)C1CCC2C1	0.0250	0.2049	0.0895	0.1541
I	T	T	80-43-3	CC(C)(OOC(C)(C)c1ccccc1)c2ccccc2	0.0000	0.2943	0.0451	0.2094
V	I	I	80-54-6	CC(C)(C)c1ccc(CC(C)C=O)cc1	0.0800	0.1301	0.2667	0.2530
I	T	I	81-15-2	O=[N+]([O-])c1c(C)c(c(C)c(c1C(C)(C)C)[N+]([O-])=O)[N+]([O-])=O	0.0300	0.0749	−0.0711	−0.1349
I	T	T	82-05-3	O=C3c4ccccc4c2cccc1cccc3c12	0.0000	0.1411	0.0152	0.0455
C	T	V	82-45-1	O=C3c1ccccc1C(=O)c2c3cccc2N	0.5000	0.2534	0.4666	0.0877
C	T	C	83-32-9	c1cc2cccc3CCc(c1)c23	0.0000	0.1077	−0.0174	−0.0173
T	T	I	84-61-7	O=C(OC1CCCCC1)c3ccccc3C(=O)OC2CCCCC2	0.6850	0.5889	0.6005	0.6626
T	T	T	85-42-7	O=C1OC(=O)C2CCCCC12	0.0350	0.1397	0.0798	0.1536
V	C	T	85-45-0	Nc1cccc([N+]([O-])=O)c1OC	0.0000	−0.0318	−0.3362	−0.1168
T	I	I	86-55-5	O=C(O)c2cccc1ccccc12	0.4000	0.1166	0.1056	0.0210

I	I	I	86-73-7	c1cccc2Cc3ccccc3c12	0.0000	0.1327	−0.0336	−0.0723
T	I	T	87-65-0	Clc1cccc(Cl)c1O	0.0000	0.2764	0.2368	0.1995
T	T	I	87-83-2	Brc1c(C)c(Br)c(Br)c(Br)c1Br	0.0700	−0.2042	−0.1680	−0.2728
T	T	T	87-84-3	BrC1C(Cl)C(Br)C(Br)C(Br)C1Br	0.0000	0.3009	0.2299	0.3546
I	T	V	87-86-5	Clc1c(O)c(Cl)c(Cl)c(Cl)c1Cl	0.0100	0.2038	0.0784	−0.0388
I	V	V	90-05-1	Oc1ccccc1OC	0.8950	0.7669	0.7291	0.6736
I	I	T	90-41-5	Nc2ccccc2c1ccccc1	0.0000	0.3994	0.3044	0.2715
C	T	I	91-01-0	OC(c1ccccc1)c2ccccc2	0.7550	0.6313	0.5690	0.5822
I	V	I	91-17-8	C1CCC2CCCCC2C1	0.0200	0.2578	0.1389	0.1571
C	I	V	91-94-1	Nc1ccc(cc1Cl)c2ccc(N)c(Cl)c2	0.0100	−0.3725	−0.1246	−0.2898
T	I	I	92-69-3	Oc1ccc(cc1)c2ccccc2	0.0000	0.5032	0.4479	0.5409
T	T	I	92-84-2	c1ccc2Sc3ccccc3Nc2c1	0.0000	0.0286	0.0085	0.0439
I	T	I	92-86-4	Brc1ccc(cc1)c2ccc(Br)cc2	0.0000	0.0153	−0.0722	−0.0701
T	I	T	93-09-4	O=C(O)c1ccc2ccccc2c1	0.8550	0.3014	0.4002	0.3856
I	I	I	93-15-2	COc1cc(ccc1OC)CC=C	0.8850	0.5863	0.3993	0.4241
I	I	T	94-52-0	[O-][N+](=O)c1cc2ncnc2cc1	0.0000	0.1354	−0.0425	0.0498
T	I	T	95-08-9	O=C(OCCOCCOCCOC(=O)C(CC)CC)C(CC)CC	0.8150	0.5351	0.5258	0.5797
T	I	I	95-16-9	c1cccc2scnc12	0.0000	0.0180	−0.0419	0.2349
V	C	I	95-49-8	Cc1ccccc1Cl	0.0000	0.0942	0.1660	0.0575
I	I	C	95-50-1	Clc1ccccc1Cl	0.0000	0.1268	0.0527	0.0524
C	V	V	95-63-6	Cc1cc(C)c(C)cc1	0.1100	0.0666	0.1246	0.0412
T	T	I	95-64-7	Cc1cc(N)ccc1C	0.0710	0.3211	0.2804	0.1866
C	V	I	95-77-2	Oc1cc(Cl)c(Cl)cc1	0.0000	0.0324	0.1445	−0.0113

(continued)

Table 4 (continued)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
I	I	C	95-78-3	Cc1cc(N)c(C)cc1	0.5000	0.2953	0.2300	0.0223
T	C	T	95-94-3	Clc1cc(Cl)c(Cl)cc1Cl	0.0000	−0.1095	−0.2421	−0.1903
I	I	V	96-18-4	ClCC(Cl)CCl	0.0000	0.2246	0.1492	0.3576
I	I	I	96-29-7	C\C(CC)=N\O	0.2470	0.3198	0.2471	0.4042
T	C	I	96-76-4	CC(C)(C)c1cc(ccc1O)C(C)(C)C	0.0000	0.1733	0.1335	0.1217
T	I	I	96-96-8	COc1ccc(N)c(c1)[N+]([O-])=O	0.0200	0.1525	−0.0238	0.0672
C	T	V	97-23-4	Oc2ccc(Cl)cc2Cc1cc(Cl)ccc1O	0.0000	0.2715	0.1095	−0.1254
I	V	I	98-08-8	FC(F)(F)c1ccccc1	0.0000	0.0859	0.0802	0.0290
V	T	V	98-15-7	FC(F)(F)c1cc(Cl)ccc1	0.0000	0.1273	0.0089	−0.0794
I	I	T	98-73-7	CC(C)(C)c1ccc(cc1)C(=O)O	0.0600	0.4517	0.5564	0.5008
T	T	I	99-54-7	Clc1ccc(cc1Cl)[N+]([O-])=O	0.0000	0.0448	−0.0671	−0.0856
T	I	I	99-82-1	CC1CCC(CC1)C(C)C	0.7600	0.4031	0.4079	0.3996
I	I	T	99-85-4	CC1=CCC(=CC1)C(C)C	0.9550	0.3983	0.4888	0.4183
I	I	I	1-40-3	C=CC1CC=CCC1	0.0000	0.2030	0.1938	0.2289
I	T	I	1-43-6	C=Cc1ccncc1	0.0000	0.1743	0.1883	0.0178
V	T	T	1-69-6	C=Cc1ccccn1	0.0000	0.3971	0.1853	0.1921
T	T	I	101-14-4	Nc2ccc(Cc1ccc(N)c(Cl)c1)cc2Cl	0.0000	0.0828	0.2070	0.0496
T	T	I	101-25-7	O=NN1CN2CN(C1)CN(C2)N=O	0.6450	0.6342	0.6416	0.6198
T	T	T	101-53-1	Oc2ccc(Cc1ccccc1)cc2	0.0000	0.5419	0.4328	0.5432
T	C	T	101-77-9	Nc2ccc(Cc1ccc(N)cc1)cc2	0.0000	−0.0741	−0.4916	−0.0895

T	T	T	102-70-5	C=CCN(CC=C)CC=C	0.0100	0.0225	−0.0126	−0.0678
I	I	T	103-23-1	O=C(CCCCC(=O)OCC(CC)CCCC)OCC(CCCC)CC	0.7050	0.4590	0.4254	0.5166
T	I	T	103-44-6	C=COCC(CCCC)CC	0.0700	0.5623	0.5440	0.4890
I	T	I	103-83-3	CN(C)Cc1ccccc1	0.0100	0.3834	0.1960	0.2342
T	C	I	105-05-5	CCc1ccc(CC)cc1	0.0000	0.1768	0.1405	0.1445
I	I	T	106-14-9	O=C(O)CCCCCCCCCCC(O)CCCCCC	0.9450	0.7807	0.6631	0.7440
V	I	V	106-91-2	CC(=C)C(=O)OCC1CO1	0.9350	0.7449	0.9127	0.6814
V	I	V	107-05-1	C=CCCl	0.6200	0.7466	0.5956	0.4531
T	I	I	107-15-3	NCCN	0.9400	0.7673	0.8423	0.7270
C	C	T	107-98-2	CC(O)COC	0.9000	0.7701	0.7998	0.7473
I	V	I	108-44-1	Cc1cc(N)ccc1	0.5000	0.5044	0.2629	0.2182
V	T	I	108-45-2	Nc1cccc(N)c1	0.0200	0.2493	−0.0968	0.1567
I	V	I	108-57-6	C=Cc1cccc(C=C)c1	0.0000	0.1739	0.0033	0.1191
T	I	I	108-60-1	CC(CCl)OC(C)CCl	0.0000	0.1188	0.2152	0.2527
T	T	T	108-86-1	Brc1ccccc1	0.0000	0.4707	0.4050	0.4384
I	I	T	108-90-7	Clc1ccccc1	0.0000	0.4295	0.1985	0.3809
I	T	I	108-93-0	OC1CCCCC1	0.9650	0.6685	0.6129	0.7070
I	I	V	108-96-3	O=C1C=CNC=C1	0.6700	0.5989	0.6672	0.5440
T	C	C	626-64-2	Oc1ccncc1	0.6700	0.5248	0.5507	0.4621
T	T	T	109-69-3	CCCCCl	0.0000	0.3859	0.4036	0.3260
T	I	T	110-00-9	c1ccco1	0.0400	0.0583	0.0060	0.0311
I	T	I	110-96-3	CC(C)CNCC(C)C	0.7950	0.7805	0.7017	0.6187
I	T	I	110-98-5	CC(O)COCC(C)O	0.0150	0.4049	0.3648	0.3266

(continued)

Table 4 (continued)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
T	T	V	111-46-6	OCCOCCO	0.9000	0.7960	0.7876	0.8218
I	I	T	111-48-8	OCCSCCO	0.1500	0.2366	0.1201	0.2370
T	I	I	111-85-3	CCCCCCCCCl	0.7750	0.3840	0.2947	0.3061
T	T	I	111-94-4	N#CCCNCCC#N	0.0200	0.3916	0.2595	0.4168
C	V	C	112-15-2	CCOCCOCCOC(C)=O	1.0000	0.8267	0.9002	0.9214
V	V	T	112-34-5	CCCCOCCOCCO	0.9100	0.7011	0.7270	0.7347
V	C	V	112-95-8	CCCCCCCCCCCCCCCCCCCC	0.8900	0.7255	0.6207	0.6167
I	C	T	115-10-6	COC	0.5000	0.4782	0.2224	0.5026
I	T	I	115-86-6	O=P(Oc1ccccc1)(Oc2ccccc2)Oc3ccccc3	0.8850	0.7834	0.8596	0.7775
T	I	T	115-96-8	ClCCOP(=O)(OCCCl)OCCCl	0.0400	0.0396	0.0114	0.3610
I	I	T	117-79-3	Nc2ccc3C(=O)c1ccccc1C(=O)c3c2	0.0100	0.0618	−0.1834	−0.1231
C	I	T	118-44-5	CCNc2cccc1ccccc12	0.0400	0.3341	0.1239	0.0985
V	C	T	118-90-1	Cc1ccccc1C(O)=O	0.9350	0.7834	0.8215	0.7914
T	T	I	119-47-1	CC(C)(C)c2cc(C)cc(Cc1cc(C)cc(c1O)C(C)(C)C)c2O	0.0000	−0.1147	−0.2982	−0.2244
I	T	T	119-53-9	OC(C(=O)c1ccccc1)c2ccccc2	0.8500	0.7547	0.6819	0.6869
I	V	T	119-93-7	Cc1cc(ccc1N)c2ccc(N)c(C)c2	0.0300	0.0028	−0.1772	−0.2528
I	I	V	120-83-2	Clc1cc(Cl)c(O)cc1	0.0000	0.0259	−0.0425	−0.1232
T	T	T	121-44-8	CCN(CC)CC	0.2950	0.1129	0.1358	0.0934
T	I	T	122-60-1	c2ccc(OCC1CO1)cc2	0.4800	0.6866	0.7294	0.5082
T	T	T	123-28-4	O=C(OCCCCCCCCCCCC)CCSCCC(=O)OCCCCCCCCCCCC	0.8200	0.7927	0.8265	0.7660

C	V	C	123-38-6	CCC=O	0.9400	0.9473	1.0267	0.9106
V	C	I	123-54-6	CC(=O)CC(C)=O	0.8350	0.8554	0.8230	0.8282
I	T	I	123-63-7	CC1OC(C)OC(C)O1	0.1200	0.4860	0.5087	0.4446
C	C	I	124-11-8	CCCCCCCC=C	0.5000	0.6581	0.5927	0.6053
I	C	I	124-17-4	CCCCOCCOCCOC(C)=O	1.0000	0.9327	0.9709	0.9106
I	T	I	127-18-4	Cl/C(Cl)=C(/Cl)Cl	0.1100	0.1516	0.1016	−0.0306
I	T	I	129-43-1	O=C3c1ccccc1C(=O)c2c3cccc2O	0.0300	0.4024	0.3942	0.3642
T	V	C	131-11-3	COC(=O)c1ccccc1C(=O)OC	0.9400	0.9401	0.8860	0.8096
I	V	I	131-17-9	O=C(OCC=C)c1ccccc1C(=O)OCC=C	0.8400	0.7541	0.6393	0.7338
V	I	T	131-57-7	O=C(c1ccc(cc1O)OC)c2ccccc2	0.0350	0.4432	0.3128	0.2790
I	I	I	132-65-0	c1cccc2sc3ccccc3c12	0.5000	0.5303	0.5240	0.3987
V	C	I	134-32-7	Nc2cccc1ccccc12	0.0000	0.3481	0.1660	0.1037
V	T	T	134-62-3	Cc1cccc(c1)C(=O)N(CC)CC	0.0000	0.4003	0.2147	0.2315
T	V	C	140-11-4	CC(=O)OCc1ccccc1	0.9400	0.8076	0.8130	0.7072
V	T	T	141-93-5	CCc1cccc(CC)c1	0.0000	0.3992	0.2233	0.2819
T	I	T	142-96-1	CCCCOCCCC	0.0350	0.7880	0.7689	0.7747
I	T	T	150-76-5	Oc1ccc(OC)cc1	0.8700	0.5177	0.4924	0.4704
I	I	T	156-43-4	CCOc1ccc(N)cc1	0.0000	0.3497	0.2341	0.2859
I	I	I	298-06-6	CCOP(=S)(S)OCC	0.0150	0.1378	−0.0229	0.0116
T	I	I	298-07-7	CCCCC(COP(O)(=O)OCC(CC)CCCC)CC	0.0850	0.1079	0.0553	0.1934
I	T	I	311-89-7	FC(F)(N(C(F)(F)C(F)(F)C(F)(F)C(F)(F)F)C(F)(F)C(F)(F)C(F)(F)C(F)(F)F)C (F)(F)C(F)(F)C(F)(F)F	0.0500	0.1332	0.0433	0.0336
T	V	V	330-54-1	Clc1ccc(NC(=O)N(C)C)cc1Cl	0.0000	0.0436	0.0344	−0.0305
T	I	V	330-55-2	Clc1ccc(NC(=O)N(C)OC)cc1Cl	0.0000	0.0241	−0.0667	−0.3003

(continued)

Table 4 (continued)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
T	T	C	462-08-8	Nc1cccnc1	0.0000	0.2133	0.0930	0.1616
V	T	I	479-27-6	Nc1cccc2cccc(N)c12	0.0000	0.1387	−0.0773	−0.1562
T	I	I	504-03-0	CC1CCCC(C)N1	0.0200	0.4616	0.4954	0.3229
T	T	I	504-24-5	Nc1ccncc1	0.0000	0.2480	0.1074	0.1222
V	C	I	504-29-0	Nc1ccccn1	0.0000	0.2113	0.0124	0.0963
C	I	T	506-52-5	CCCCCCCCCCCCCCCCCCCCCCCCCO	0.7500	0.7404	0.6894	0.7423
I	I	T	510-15-6	OC(c1ccc(Cl)cc1)(c2ccc(Cl)cc2)C(=O)OCC	0.0000	0.1338	0.0060	0.1634
I	T	I	512-56-1	O=P(OC)(OC)OC	0.0000	0.1643	0.0077	0.1215
I	T	T	527-60-6	Cc1cc(C)c(O)c(C)c1	0.0700	0.1840	0.2306	0.2406
I	I	T	532-03-6	NC(=O)OCC(O)COc1ccccc1OC	0.0450	0.4539	0.4377	0.4551
I	I	T	535-77-3	Cc1cccc(c1)C(C)C	0.0000	0.6225	0.6041	0.4826
T	I	T	538-75-0	N(=C=N\C1CCCCC1)\C2CCCCC2	0.5000	0.4851	0.5110	0.5105
I	V	I	541-41-3	ClC(=O)OCC	0.8350	0.8035	0.6775	0.9404
V	I	C	541-73-1	Clc1cccc(Cl)c1	0.0000	0.2653	−0.0275	0.0430
I	T	T	552-30-7	O=C(O)c1ccc2C(=O)OC(=O)c2c1	0.9500	0.7439	0.7853	0.5869
I	V	I	554--7	Clc1cc(Cl)c(N)cc1	0.0000	0.0670	0.0927	0.0735
I	I	I	563-47-3	C=C(C)CCl	0.9800	0.7526	0.7141	0.4618
T	V	T	576-24-9	Oc1cccc(Cl)c1Cl	0.0000	0.1575	0.2454	0.0779
I	T	T	579-10-2	CN(C(C)=O)c1ccccc1	0.0100	0.3657	0.1425	0.1830
V	C	V	583-78-8	Clc1ccc(Cl)c(O)c1	0.0500	0.0259	−0.0425	−0.1232

T	I	I	591-27-5	Nc1cccc(O)c1	0.0000	0.2473	0.2067	0.2255
V	V	T	591-35-5	Oc1cc(Cl)cc(Cl)c1	0.0000	0.2877	0.0904	0.0179
T	I	T	592-41-6	C=CCCCC	0.8250	0.6775	0.6117	0.6216
I	I	I	603-11-2	O=[N+]([O-])c1cccc(c1C(=O)O)C(=O)O	0.0000	0.3873	0.3410	0.4206
T	I	T	606-28-0	COC(=O)c2ccccc2C(=O)c1ccccc1	0.0100	0.3439	0.4277	0.2826
I	C	I	608-93-5	Clc1cc(Cl)c(Cl)c(Cl)c1Cl	0.0000	0.0941	−0.0600	−0.0716
C	T	V	609-89-2	Oc1c(cc(Cl)cc1Cl)[N+]([O-])=O	0.0000	0.4210	0.1193	0.2070
T	T	I	611-21-2	Cc1ccccc1NC	0.0100	0.3216	0.3211	0.3897
C	T	I	614-80-2	Oc1ccccc1NC(C)=O	0.7900	1.1219	0.9794	0.9432
V	V	V	615-74-7	Cc1ccc(Cl)c(O)c1	0.0550	0.0409	0.1690	−0.0545
V	V	I	618-36-0	CC(N)c1ccccc1	0.0600	0.4359	0.2441	0.2371
C	C	I	622-57-1	CCNc1ccc(C)cc1	0.0200	0.0126	0.1257	0.0225
I	I	T	626-17-5	N#Cc1cccc(C#N)c1	0.0000	0.1817	−0.1905	0.0253
I	T	T	626-67-5	CN1CCCCC1	0.0100	0.1926	0.2023	0.2396
C	V	T	629-73-2	CCCCCCCCCCCCCC=C	0.6600	0.6128	0.5481	0.5673
I	I	C	634-66-2	Clc1ccc(Cl)c(Cl)c1Cl	0.0000	−0.1008	−0.1877	−0.1672
V	I	I	634-93-5	Clc1cc(Cl)cc(Cl)c1N	0.0000	0.0662	−0.1241	0.0400
T	I	I	674-82-8	C=C1CC(=O)O1	0.9850	0.7790	0.7998	0.6823
T	T	I	680-31-9	CN(C)P(=O)(N(C)C)N(C)C	0.5000	0.4935	0.5021	0.5520
T	T	T	696-44-6	Cc1cc(NC)ccc1	0.0000	0.3453	0.3336	0.1526
C	I	I	708-06-5	O=Cc1c2ccccc2ccc1O	0.6550	0.4263	0.5097	0.4771
I	I	C	732-26-3	CC(C)(C)c1cc(cc(c1O)C(C)(C)C)C(C)(C)C	0.0000	0.0753	−0.0048	−0.0575
I	I	I	782-74-1	Clc2ccccc2NNc1ccccc1Cl	0.0000	0.1408	−0.0310	−0.1473

(continued)

Table 4 (continued)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
I	V	I	818-61-1	C=CC(=O)OCCO	0.7800	0.7895	0.8175	0.7677
I	T	T	828--2	O=C(C)OC1CC(C)OC(C)O1	0.7950	0.6894	0.6606	0.5926
I	I	I	872-50-4	O=C1CCCN1C	0.7300	0.5946	0.6057	0.6025
I	T	I	920-66-1	FC(F)(F)C(O)C(F)(F)F	0.0000	0.1616	0.0049	−0.2572
V	V	I	927-49-1	CCCCCC(=O)CCCCC	0.7050	0.9608	0.9045	0.9325
T	T	I	947-04-6	O=C1CCCCCCCCCCCN1	0.0000	0.5694	0.5814	0.5460
I	T	V	1129-41-5	O=C(Oc1cc(C)ccc1)NC	0.3850	0.3346	0.2564	0.1134
T	T	T	1141-38-4	O=C(O)c1ccc2cc(ccc2c1)C(=O)O	0.0000	0.4468	0.5104	0.4585
T	I	T	1212-29-9	S=C(NC1CCCCC1)NC2CCCCC2	0.0000	0.0097	−0.0170	0.0021
I	T	T	1241-94-7	CCC(CCCC)COP(=O)(Oc1ccccc1)Oc2ccccc2	0.0100	0.2610	0.2179	0.0189
T	T	I	1460-02-2	CC(C)(C)c1cc(cc(c1)C(C)(C)C)C(C)(C)C	0.0000	−0.1499	−0.2257	−0.3682
C	I	T	1461-25-2	CCCC[Sn](CCCC)(CCCC)CCCC	0.0000	0.2791	0.0409	0.1594
I	T	T	1477-55-0	NCc1cccc(CN)c1	0.2200	0.3739	0.3128	0.3561
I	I	T	1502-22-3	O=C2CCCCC2C=1CCCCC=1	0.0100	0.4532	0.4364	0.4242
T	V	T	1570-64-5	Cc1cc(Cl)ccc1O	0.0000	0.2161	0.2704	0.0801
I	I	I	1712-70-5	CC(=C)c1ccc(Cl)cc1	0.0000	0.4406	0.2915	0.1962
V	C	V	2050-76-2	Clc1cc(Cl)c(O)c2ccccc12	0.0000	−0.2755	−0.3221	−0.2943
I	T	I	2104-64-5	S=P(OCC)(Oc1ccc(cc1)[N+]([O-])=O)c2ccccc2	0.0300	0.1654	0.0295	0.0164
T	T	I	2173-57-1	CC(C)COc1ccc2ccccc2c1	0.0100	0.2954	0.4419	0.4441
T	I	I	2216-69-5	COc2cccc1ccccc12	0.0000	0.3231	0.2189	0.2156

C	V	C	2243-27-8	CCCCCCCCC#N	0.6700	0.6731	0.9084	0.6293
V	C	I	2243-62-1	Nc2cccc1c2cccc1N	0.0000	0.1342	−0.1077	0.1277
I	I	I	2305-26-2	OC(=O)C1CC=CCC1C(=O)O	0.0000	0.5145	0.3242	0.5159
I	T	T	2498-66-0	O=C3c2c(ccc1ccccc12)C(=O)c4ccccc34	0.0100	0.1393	0.0236	0.0440
V	T	I	2597-03-7	O=C(OCC)C(SP(=S)(OC)OC)c1ccccc1	0.0150	0.2092	0.0128	−0.0524
C	V	I	2627-35-2	OP(O)(=O)OCCCCCCCCCCCC	0.8350	0.7452	0.7227	0.5665
I	I	I	2631-40-5	O=C(Oc1ccccc1C(C)C)NC	0.0000	0.2790	0.2672	0.2216
I	I	C	2655-14-3	O=C(Oc1cc(C)cc(C)c1)NC	0.0100	0.2363	0.0853	−0.0360
C	C	I	2668-47-5	CC(C)(C)c1cc(cc(c1O)C(C)(C)C)c2ccccc2	0.0000	0.0979	0.0459	−0.0246
C	V	T	2814-20-2	O=C1C=C(C)NC(=N1)C(C)C	0.0000	0.1349	0.1194	0.0253
I	T	I	2814-20-2	Cc1cc(O)nc(n1)C(C)C	0.0000	0.0925	0.0909	0.1489
T	T	T	2814-20-2	O=C1C=C(C)NC(=N1)C(C)C	0.0000	0.1349	0.1194	0.0253
T	T	I	2885--9	CCCCCCCCCCCCCCCCCCS	0.0000	0.0233	−0.0009	−0.0511
T	T	T	3090-36-6	CCCC[Sn](CCCC)(OC(=O)CCCCCCCCCCC)CCCC	0.0350	0.0439	0.0462	0.0966
T	T	T	3229--3	BrCC(CBr)(CBr)CBr	0.2900	0.2263	0.0815	0.1717
T	I	I	3296-90-0	OCC(CBr)(CBr)CO	0.1800	0.2182	0.4378	0.2243
V	I	T	3319-31-1	CCC(CCCC)COC(=O)c1cc(C(=O)OCC(CC)CCCC)c(cc1)C(=O)OCC(CC) CCCC	0.0420	0.1165	0.0241	0.0090
T	T	T	3370-35-2	O=C(CCCCCCCCCCCCCCCCC)NCO	0.8600	0.6048	0.4871	0.5094
I	I	T	3380-34-5	Clc2cc(Cl)ccc2Oc1ccc(Cl)cc1O	0.0000	0.2543	0.0925	0.1369
T	I	T	3766-81-2	O=C(Oc1ccccc1C(C)CC)NC	0.5000	0.2598	0.1863	0.2065
T	I	I	4101-68-2	BrCCCCCCCCCCBr	0.1600	0.3225	0.3425	0.4270
C	T	I	4130-42-1	CC(C)(C)c1cc(cc(c1O)C(C)(C)C)CC	0.0000	0.1596	0.1585	0.1271
I	T	I	5124-25-4	O=S(=O)(Nc1ccccc1)c3cc(c(Nc2ccccc2)cc3)[N+]([O-])=O	0.0000	0.0789	0.0101	−0.0292

(continued)

Table 4 (continued)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
T	T	T	5131-66-8	CC(O)COCCCC	0.8850	0.5414	0.5489	0.4823
T	I	T	5510-99-6	Oc1c(cccc1C(C)CC)C(C)CC	0.0300	0.3066	0.1412	0.3842
I	T	T	6731-36-8	CC(C)(C)OOC1(OOC(C)(C)C)CC(C)(C)CC(C)C1	0.0000	0.0662	−0.0512	−0.0655
I	I	V	7525-62-4	CCc1cccc(C=C)c1	0.0000	0.2605	0.1252	0.2107
T	I	T	10315-98-7	CC(C)CN1CCOCC1	0.5000	0.4604	0.4340	0.4619
T	T	I	10541-83-0	CNc1ccc(cc1)C(=O)O	0.1400	0.2184	0.3634	0.3118
T	I	I	10605-21-7	O=C(OC)Nc1nc2ccccc2n1	0.0000	0.0077	−0.0432	−0.1997
I	C	T	13116-53-5	ClCC(Cl)(Cl)CCl	0.0100	0.0149	0.1223	0.0902
I	T	I	13358-11-7	CC(C)C13CCC(C)(C=C1)C2C(=O)N(CC(CC)CCCC)C(=O)C23	0.0100	0.1663	0.0052	0.0109
I	T	I	13674-84-5	ClCC(C)OP(=O)(OC(C)CCl)OC(C)CCl	0.0000	0.1797	0.0619	0.4755
T	T	I	13674-87-8	ClCC(OP(=O)(OC(CCl)CCl)OC(CCl)CCl)CCl	0.0200	−0.0077	−0.0197	0.2962
I	T	I	13952-84-6	CC(N)CC	0.8850	0.5773	0.6845	0.4432
T	T	C	14816-18-3	CCOP(=S)(O\N=C(/C#N)c1ccccc1)OCC	0.0550	0.0711	0.0547	−0.3304
T	I	T	15104-61-7	ClC(Cl)C(Cl)C(Cl)Cl	0.0000	0.0461	−0.0359	0.0903
T	I	T	16091-18-2	O=C1O[Sn](CCCCCCCC)(OC(=O)C=C1)CCCCCCCC	0.0300	0.0394	0.0691	0.1392
T	I	T	16219-75-3	C\C=C2\CC1C=CC2C1	0.0000	0.0207	−0.2032	−0.1717
I	T	C	19438-60-9	O=C1OC(=O)C2CC(C)CCC12	0.0000	0.0883	0.1013	−0.0443
T	T	I	20241-76-3	[O-][N+](=O)c4ccc(O)c2c4C(=O)c1c(ccc(O)c1C2=O)Nc3ccccc3	0.0000	0.0235	−0.0109	−0.0463
C	T	I	21564-17-0	N#CSCSc1nc2ccccc2s1	0.0000	−0.2735	0.0079	−0.1096
T	I	T	25395-31-7	O=C(C)OC(CO)COC(=O)C	0.9250	0.7062	0.7194	0.7431

I	I	I	28623-46-3	CCCCCCCCCCCCCCCCCCCC#N	0.9250	0.6083	0.8447	0.5750
C	I	I	31732-71-5	CCCC[Sn](CCCC)(OC(=O)C(Br)C(Br)C(=O)O[Sn](CCCC)(CCCC)CCCC)CCCC	0.0100	−0.5419	−0.0869	−0.2306
T	I	V	32360-05-7	C=C(C)C(=O)OCCCCCCCCCCCCCCCCCC	0.8700	0.7337	0.7665	0.6946
T	T	I	40690-89-9	[O-][N+](=O)c1ccc(cc1)/N=N/c2ccc(cc2)N(CCOC(=O)c3ccccc3)CCC#N	0.0700	0.1152	0.0916	0.0157
C	I	I	41122-70-7	N#Cc1ccc(cc1)c2ccc(CCCCCC)cc2	0.0250	−0.1194	0.0483	0.0102
I	I	T	59722-76-8	Brc1ccc(cc1)Oc4cc(O)c3c(C(=O)c2ccccc2C3=O)c4N	0.5000	0.5154	0.4966	0.5132
I	C	C	95-53-4	Cc1ccccc1N	0.6540	0.6988	0.5191	0.4665
V	C	C	109-89-7	CCNCC	0.6900	1.0444	0.9972	0.7101
I	I	C	111-92-2	CCCCNCCCC	0.9550	0.7402	0.7388	0.6287
I	T	I	24851-98-7	COC(=O)CC1CCC(=O)C1CCCCC	0.9850	0.8927	0.9877	1.0932
T	I	T	118-79-6	Brc1cc(Br)cc(Br)c1O	0.4900	0.4874	0.3833	0.4968
I	T	T	79-46-9	CC(C)[N+]([O-])=O	0.1100	0.2941	0.2893	0.2455
I	I	T	91-20-3	c1cccc2ccccc12	0.0200	0.3553	0.2785	0.1920
C	T	C	61-82-5	Nc1nncn1	0.0000	−0.2156	−0.0068	−0.2267
V	C	T	128-37-0	CC(C)(C)c1cc(C)cc(c1O)C(C)(C)C	0.0450	0.1442	0.2454	0.0916
T	I	T	1-41-4	CCc1ccccc1	1.0000	0.6603	0.5779	0.5079
T	I	I	1-26-5	OC(=O)c1ccc(cn1)C(=O)O	0.0000	0.2556	0.1602	0.1748
I	T	T	119-56-2	OC(c1ccc(Cl)cc1)c2ccccc2	0.5000	0.2144	0.1555	0.1339
T	T	T	132-64-9	c1cccc2oc3ccccc3c12	0.0000	0.0230	0.0046	0.0024
T	V	V	208-96-8	c1cc2cccc3C=Cc(c1)c23	0.0000	−0.0310	−0.1155	−0.0884
T	T	I	585-07-9	CC(=C)C(=O)OC(C)(C)C	0.3200	0.5398	0.4326	0.4556
T	T	T	764-13-6	C/C(C)=C\C=C(/C)C	0.0350	0.4352	0.3959	0.4160
I	I	I	791-31-1	O[Si](c1ccccc1)(c2ccccc2)c3ccccc3	0.0000	0.1087	−0.0290	−0.0426

(continued)

Table 4 (continued)

Set	Set	Set	CAS	SMILES	Expr	Eq. 8	Eq. 9	Eq. 10
I	I	I	1623-19-4	C=CCOP(=O)(OCC=C)OCC=C	0.2050	0.1007	0.1250	0.0330
I	T	I	1726-23-4	O=C(OCCCC)c1cc(C(=O)OCCCC)c(cc1)C(=O)OCCCC	0.8850	0.8618	0.6637	0.7648
V	I	V	1805-32-9	Clc1ccc(CO)cc1Cl	0.0000	−0.1312	−0.0163	−0.0333
I	T	T	2082-80-6	CCCCCCCCCCCCCCCCCCOP(OCCCCCCCCCCCCCCCCCC)OCCCCCCCCCCCCCCCCCC	0.6650	0.6567	0.7399	0.6717
I	I	T	4821-19-6	Oc1c(cccc1C2CCCCC2)C3CCCCC3	0.0000	0.0577	−0.0825	−0.0477
C	C	V	124-28-7	CN(C)CCCCCCCCCCCCCCCCCC	0.5900	0.5581	0.4715	0.4755
I	I	I	108-87-2	CC1CCCCC1	0.0000	0.5798	0.6066	0.5709
I	T	T	107-39-1	C=C(C)CC(C)(C)C	0.0000	0.4658	0.2846	0.3376
V	T	C	59-50-7	Oc1ccc(Cl)c(C)c1	0.0000	−0.0779	0.0468	−0.0094
V	C	I	23778-52-1	COCCOCCOCCOCCOCCO	0.5450	0.6100	0.7199	0.6689
I	C	I	26967-76-0	CC(C)c3ccccc3OP(=O)(Oc1ccccc1C(C)C)Oc2ccccc2C(C)C	0.0000	0.0997	−0.0010	−0.0413
T	I	T	60628-17-3	CCCCCCCCCCCCCOP(Oc1ccccc1)Oc2ccccc2	0.1950	0.2229	0.1755	0.1933
T	T	V	29761-21-5	CC(C)CCCCCCCOP(=O)(Oc1ccccc1)Oc2ccccc2	0.5000	0.3579	0.2607	−0.0578
T	I	I	34375-28-5	OCCNCO	0.6800	0.7357	0.8002	0.6516
I	I	I	1779-48-2	OP(O)c1ccccc1	0.0800	0.1731	0.0384	0.1735
I	I	T	15205-57-9	O(Cc1ccccc1)P(OCc2ccccc2)OCc3ccccc3	0.7900	0.7732	0.7493	0.7511
I	I	T	61968-52-3	Clc2cc(ccc2/N=N/c1ccc(cc1NC(=O)CC)N(CCOC(C)=O)CCOC(C)=O)[N+]([O-])=O	0.0000	0.0442	−0.0500	0.2609
I	T	C	149-44-0	OCS(=O)O	1.0000	0.9266	1.0144	0.8422
I	T	T	577-11-7	O=S(=O)(O)C(CC(=O)OCC(CC)CCCC)C(=O)OCC(CC)CCCC	0.0450	0.1573	0.0274	0.0908

T	V	I	35860-37-8	CC(C)c2cccc1c2cc(cc1C(C)C)C(C)C	0.0000	0.0406	0.0291	−0.0789
I	I	T	28299-29-8	O=C(O)C(CCCCCCCC\C=C\CCCCCCCC)CC(=O)O	0.0700	0.4949	0.4493	0.5035
T	I	T	32669-06-0	ClCCOC(c1ccccc1)c2ccccc2	0.0000	0.0841	0.0176	0.2782
V	V	I	13540-50-6	Cc2cc(Cc1ccccc1)c(C)cc2	0.0100	0.4001	0.2710	0.2108
I	I	T	5586-15-2	c3cccc4ccc(SSc1cc2ccccc2cc1)cc34	0.0000	0.1175	−0.0456	0.0094
T	I	T	59365-60-5	OC(CO)c1ccccc1Cl	0.0000	0.1055	0.1204	0.0849
T	T	T	10496-18-1	CCCCCCCCCCSSCCCCCCCCCC	0.0350	0.0407	0.0433	0.0195
T	V	I	1631-58-9	CN(C)C1CSSC1	0.0000	0.0190	−0.0344	−0.0240
T	T	T	24019-05-4	Clc3ccc(NC(=O)Nc2cc(Cl)ccc2Oc1ccc(Cl)cc1S(=O)(=O)O)cc3Cl	0.0000	0.0244	−0.0044	0.0176
T	T	T	56113-42-9	Clc1c(c(c(Cl)c(Cl)c1Cl)C(=O)O)C(N)=O	0.5000	0.2446	0.5163	−0.0325
T	T	T	4051-66-5	CCCCCCCCCCCCCCCCCCNC(=O)NCCCCCCCCCCCCCCCCCC	0.0000	0.1422	0.2685	0.1948
T	I	I	4883-72-1	ON(N=O)C1CCCCC1	0.0000	0.0197	−0.0067	0.0260
T	I	T	7267-11-0	O=C(O)c1ccc(Cl)c2c1c(ccc2Cl)C(=O)O	0.0000	0.0063	−0.0512	−0.0493
T	T	I	60782-58-3	CCCCCC[Si](O)(CCCCCC)CCCCCC	0.5000	0.5034	0.4935	0.4140
C	V	V	14130-05-3	CCCCCCCCCCCCCCCCCCCCN	0.6150	0.6860	0.6208	0.5917
I	I	C	19045-79-5	OP(O)(=O)OCCCCCCCC	0.7450	0.7711	0.7482	0.5882
T	T	I	26076-28-8	O=Nc5c6ccccc6ccc5O[Co](Oc2ccc1ccccc1c2N=O)Oc4ccc3ccccc3c4N=O	0.0000	0.0187	0.0024	−0.0447
T	T	I	50853-70-8	CC2C1C=CC2C(C1C(=O)O)C(=O)O	0.0000	−0.0180	0.1089	0.0342
C	C	T	63148-74-3	OCS(=O)(=O)O	1.0000	0.9787	1.1821	1.0303

Table 5
Experimental and predicted values of biodegradability for three random splits into the training (T), invisible training (I), calibration (C), and validation (V) sets (dataset 2, $n = 59$)

Set	Set	Set	ID	SMILES	Expr	Eq. 11	Eq. 12	Eq. 13
I	T	I	TMP	CC(CN(C)C)CN1c2ccccc2CCc3ccccc13	0.0010	−0.2515	−0.0431	−0.1989
V	C	T	PTP309a	CC(CN(C)C)CN1c2ccccc2CC(=O)c3ccccc13	0.0180	0.0085	0.0629	0.0157
I	C	I	PTP311-1	CC(CN(C)C)CN1c2ccccc2CC(O)c3ccccc13	0.0030	−0.0540	0.0472	−0.0362
V	T	V	PTP313-1	CC(CN(C)C)CN(c1ccccc1)c2ccccc2CCO	0.0200	−0.0414	0.0206	−0.0677
I	T	T	PTP313-2	CC(CN(C)C)CN(c1ccccc1C)c2ccccc2CO	0.0210	−0.0694	−0.0068	−0.0302
C	T	V	PTP327-1	CC(CN(C)C)CN1c2cccc(O)c2CCc3cccc(O)c13	0.1680	0.1557	0.1793	0.2057
C	T	C	PTP327-2	CC(CN(C)C)CN1c2ccc(O)cc2CCc3cccc(O)c13	0.1700	0.1467	0.1796	0.1931
C	I	T	PTP327-3	CC(CN(C)C)CN1c2cc(O)ccc2CCc3cccc(O)c13	0.2270	0.1582	0.1849	0.1796
I	V	V	PTP327-4	CC(CN(C)C)CN1c2c(O)cccc2CCc3cccc(O)c13	0.1500	0.1048	0.1613	0.1740
I	I	I	PTP327-5	CC(CN(C)C)C(O)N1c2ccccc2CCc3cccc(O)c13	0.2110	0.2296	0.1707	0.2724
C	V	C	PTP327-6	CC(CN(C)C)CN1c2cccc(O)c2CCc3ccc(O)cc13	0.1930	0.1384	0.1528	0.1560
I	I	V	PTP327-7	CC(CN(C)C)CN1c2cc(O)ccc2CCc3ccc(O)cc13	0.2020	0.1410	0.1584	0.1300
I	T	T	PTP327-8	CC(CN(C)C)CN1c2ccc(O)cc2CCc3ccc(O)cc13	0.1940	0.1294	0.1530	0.1434
T	I	T	PTP327-9	CC(CN(C)C)C(O)N1c2ccccc2CCc3ccc(O)cc13	0.1870	0.2831	0.1943	0.2779
I	V	I	PTP327-10	CC(CN(C)C)CN1c2ccc(O)cc2CCc3c(O)cccc13	0.1870	0.1269	0.1475	0.1695
V	V	V	PTP327-11	CC(CN(C)C)CN1c2ccc(O)cc2CCc3cc(O)ccc13	0.1900	0.1179	0.1477	0.1569
T	T	T	PTP327-12	CC(CN(C)C)C(O)N1c2ccccc2CCc3cc(O)ccc13	0.2150	0.2706	0.2153	0.2872
T	C	T	PTP327-13	CC(CN(C)C)CN1c2cccc(O)c2CCc3c(O)cccc13	0.1850	0.1358	0.1473	0.1821
V	C	C	PTP327-14	CC(CN(C)C)C(O)N1c2ccccc2CCc3c(O)cccc13	0.2150	0.2805	0.1888	0.3040

I	V	T	PTP327-15	CC(CN(C)C)CN1c2ccccc2C(O)C(O)c3ccccc13	0.0030	−0.0305	0.0966	0.0466
C	C	V	PTP327-16	CC(CN(C)C)CN1c2ccccc2C(O)Cc3c(O)cccc13	0.0950	0.0904	0.1255	0.1206
T	I	I	PTP327-17	CC(CN(C)C)CN1c2ccc(O)cc2CC(O)c3ccccc13	0.0960	0.0716	0.1266	0.0683
T	T	V	PTP327-18	CC(CN(C)C)CN1c2ccccc2CC(O)c3c(O)cccc13	0.0970	0.1118	0.1259	0.1038
I	V	C	PTP327-19	CC(CN(C)C)CN1c2ccccc2C(O)Cc3cccc(O)c13	0.0750	0.1188	0.1599	0.1281
I	I	C	PTP327-20	CC(CN(C)C)CN1c2ccccc2C(O)Cc3ccc(O)cc13	0.1490	0.1339	0.1446	0.1348
T	V	C	PTP327-21	CC(CN(C)C)CN1c2ccccc2CC(O)c3cc(O)ccc13	0.0960	0.1145	0.1437	0.0839
T	I	T	PTP327-22	CC(CN(C)C)CN1c2ccccc2CC(O)c3ccc(O)cc13	0.0710	0.1594	0.1339	0.1311
C	I	I	PTP327-23	CC(CN(C)C)CN1c2ccccc2CC(O)c3cccc(O)c13	0.0760	0.1014	0.1321	0.1087
T	I	I	PTP327-24	CC(CN(C)C)C(O)N1c2ccccc2CC(O)c3ccccc13	0.3660	0.2576	0.1791	0.2592
V	T	I	PTP329-1	CC(CN(C)C)CN(c1ccccc1CO)c2ccccc2CO	0.0210	0.0012	0.0487	−0.0063
I	T	I	PTP134	CN(C)CC(O)CCO	0.3470	0.3019	0.3467	0.2880
I	I	T	PTP196	C1Cc2ccccc2Nc3ccccc13	0.0000	0.1336	0.1139	0.1718
T	I	T	PTP210-1	O=C1Cc2ccccc2Nc3ccccc13	0.6290	0.5641	0.2951	0.4767
I	I	C	PTP212-1	OC1Cc2ccccc2Nc3ccccc13	0.5540	0.5101	0.2717	0.3978
C	C	V	PTP212-2	Oc1cccc2Nc3ccccc3CCc12	0.2700	0.3442	0.1980	0.3353
C	V	T	PTP212-3	Oc1cccc2CCc3ccccc3Nc12	0.2770	0.2308	0.1908	0.3085
T	I	I	PTP212-4	Oc1ccc2CCc3ccccc3Nc2c1	0.1880	0.1958	0.1657	0.2512
T	I	I	PTP212-5	Oc1ccc2Nc3ccccc3CCc2c1	0.2700	0.2998	0.1789	0.2745
T	I	C	PTP212-6	ON1c2ccccc2CCc3ccccc13	0.0000	−0.0049	0.1062	0.1449
V	T	T	PTP250	CC(C)CN1c2ccccc2C=Cc3ccccc13	0.0200	−0.0763	0.0364	−0.0240
T	T	T	PTP268-1	CC(C)CN1c2ccccc2CC(O)c3ccccc13	0.0000	0.0158	0.0758	0.0203
I	C	V	PTP268-2	CC(C)CN1c2ccccc2CCc3c(O)cccc13	0.1260	0.0386	0.0855	0.0651

(continued)

Table 5 (continued)

Set	Set	Set	ID	SMILES	Expr	Eq. 11	Eq. 12	Eq. 13
V	I	I	PTP268-3	CC(C)CN1c2ccccc2CCc3cccc(O)c13	0.1020	0.0585	0.1176	0.0887
T	C	T	PTP268-4	CC(C)CN1c2ccccc2CCc3ccc(O)cc13	0.1030	0.0412	0.0911	0.0390
V	T	T	PTP268-5	CC(C)CN1c2ccccc2CCc3cc(O)ccc13	0.1260	0.0297	0.0858	0.0525
T	T	I	PTP268-6	CC(C)C(O)N1c2ccccc2CCc3ccccc13	0.1290	0.0383	0.1152	0.0381
T	C	I	PTP405-1	OC1Cc2ccccc2N(N3c4ccccc4CCc5ccccc35)c6ccccc16	0.2780	0.2910	0.1979	0.2499
T	V	T	PTP405-2	Oc1cccc2N(N3c4ccccc4CCc5ccccc35)c6ccccc6CCc12	0.1300	0.1262	0.1466	0.1805
C	V	T	PTP405-3	Oc1ccc2N(N3c4ccccc4CCc5ccccc35)c6ccccc6CCc2c1	0.1300	0.1122	0.1656	0.1445
T	T	I	PTP405-4	Oc1ccc2CCc3ccccc3N(N4c5ccccc5CCc6ccccc46)c2c1	0.1730	0.2095	0.1793	0.1845
T	I	T	PTP405-5	Oc1cccc2CCc3ccccc3N(N4c5ccccc5CCc6ccccc46)c12	0.2970	0.2412	0.1921	0.2533
C	T	C	PTP247-1	CNCC(C)CN1C=CC(O)Cc2ccccc12	0.0940	0.1565	0.1356	0.1802
V	C	I	PTP247-2	CNCC(C)CN1C=CCC(O)c2ccccc12	0.0960	0.1031	0.1174	0.1263
V	I	T	PTP247-3	CNCC(C)CN1C=C(O)CCc2ccccc12	0.0960	0.1843	0.1340	0.1532
C	V	C	PTP247-4	CNCC(C)CN1C=CCCc2cc(O)ccc12	0.1970	0.2043	0.1774	0.1877
V	C	I	PTP247-5	CNCC(C)CN1C=CCCc2c(O)cccc12	0.1970	0.1265	0.1436	0.1417
T	T	I	PTP247-6	CNCC(C)CN1C(O)=CCCc2ccccc12	0.0340	0.1215	0.0742	0.0829
V	T	V	PTP247-7	CNCC(C)CN1C=CCCc2ccc(O)cc12	0.2040	0.1719	0.1662	0.1313
I	T	T	PTP247-8	CNCC(C)CN1C=CCCc2cccc(O)c12	0.2270	0.1805	0.1733	0.1459

Table 6
The comparison of roles of molecular features with significant prevalence in models of biodegradability for dataset 1 and dataset 2 (first splits). The T_{445}, I_{445}, C_{445} are the numbers of a feature in training, invisible training, and calibration sets, respectively. The T_{59}, I_{59}, C_{59} are the similar data on dataset 2. Plus means positive correlation weights. Minus means negative correlation weights

ID	Feature	1	2	3	1	2	3	T_{445}	I_{445}	C_{445}	T_{59}	I_{59}	C_{59}
	Equivalent manner												
1	C5......0...	+	+	+	+	+	+	149	153	53	20	16	11
2	PT2-C...2...	+	+	+	+	+	+	142	143	55	20	16	11
3	EC2-C...9...	+	+	+	+	+	+	79	74	28	20	16	11
4	NNC-O...110.	+	+	+	+	+	+	76	76	38	19	14	11
5	EC2-C...10..	+	+	+	+	+	+	55	69	25	15	10	11
6	EC2-O...6...	+	+	+	+	+	+	25	23	10	12	9	8
7	3..........	+	+	+	+	+	+	11	15	3	19	13	9
8	C..........	–	–	–	–	–	–	142	139	52	20	16	11
9	NNC-C...220.	–	–	–	–	–	–	131	123	49	20	16	11
10	(..........	–	–	–	–	–	–	119	125	40	16	14	9
11	C...(.......	–	–	–	–	–	–	99	105	31	12	14	8
12	O...(.......	–	–	–	–	–	–	70	77	24	12	12	8
13	NNC-C...211.	–	–	–	–	–	–	67	51	27	11	14	8
14	NNC-C...330.	–	–	–	–	–	–	67	82	23	20	15	11
15	EC2-C...12..	–	–	–	–	–	–	56	62	17	20	15	11
16	N..........	–	–	–	–	–	–	48	46	16	20	16	11
	Opposite												
1	c...2.......	+	+	+	–	–	–	35	32	12	20	15	11
2	N...C.......	+	+	+	–	–	–	28	18	8	11	14	8
3	O..........	–	–	–	+	+	+	107	111	43	20	14	11
4	EC2-C...11..	–	–	–	+	+	+	64	66	24	18	10	9
5	EC2-C...17..	–	–	–	+	+	+	33	37	12	12	9	7
6	N...(.......	–	–	–	+	+	+	30	22	3	13	12	7
7	EC2-C...18..	–	–	–	+	+	+	22	28	9	15	8	5
8	EC2-C...19..	–	–	–	+	+	+	20	25	6	14	11	11
9	C...2.......	–	–	–	+	+	+	11	13	2	15	12	7

Table 7
The number of molecular features which have equivalents and contradict effects to biodegradability for dataset 1 ($n = 445$) and dataset 2 ($n = 59$)

	Action	445-1	445-2	445-3	59-1	59-2	59-3
445-1	Equivalent Opposite	72 0	46 3	47 7	16 9	15 9	15 10
445-2	Equivalent Opposite		56 0	40 5	15 9	15 7	14 10
445-3	Equivalent Opposite			64 0	14 11	15 8	14 11
59-1	Equivalent Opposite				46 0	33 5	38 3
59-2	Equivalent Opposite					44 0	37 5
59-3	Equivalent Opposite						46 0

cases or they are promoters of decrease of biodegradability for both the cases. However, there are also nine features with opposite actions, i.e., these nine features are promoters of increase of biodegradability for dataset 1 and promoters of decrease of biodegradability for dataset 2 and vice versa.

Table 7 contains number of features, which have equivalent role, and opposite role in building up models of biodegradability. One can see, the numbers of features with opposite actions for splits related to the same dataset are less than the number of the above-mentioned features for splits related to different datasets.

Table 8 contains the list of molecular features, which are stable promoters of increase or decrease of biodegradability for both datasets and for all splits.

7 The Correlation Contributions of Rings

The QSAR models for dataset 1 have apparent improvement if correlation weights of the above-described C5 and C6 are involved in the modeling process. However, these molecular features cannot improve the predictive potential for models in the case of dataset 2. The possible cause of such a situation is the different structural circumstances for dataset 1 and dataset 2. Dataset 1 contains both molecular structures with rings and acyclic molecular structures (Table 4) whereas all molecular structures of dataset 2 contain rings (Table 5).

Table 8
The number of molecular features which have equivalents and contradict effects to biodegradability for dataset 1 (n = 445) and dataset 2 (n = 59)

Feature	Comment	Influence for biodegradability
PT2.C...2...	Two paths of length 2 started from carbon vertex in HSG	Increase
C6...A..2...	Presence of two six-members aromatic rings	Increase
EC2-C...9...	Presence of carbon vertex with extended connectivity of second order equal to 9	Increase
EC2-C...4...	Presence of carbon vertex with extended connectivity of second order equal to 4	Increase
NNC-C...220.	Presence of carbon vertex with NNC = 220	Increase
EC2-C...11..	Presence of carbon vertex with extended connectivity of second order equal to 11	Decrease
EC2-C...18..	Presence of carbon vertex with extended connectivity of second order equal to 18	Decrease
O..........	Presence of oxygen atoms	Decrease
N...C.......	Presence of connected nitrogen and carbon atoms	Decrease

8 Comparison with Models for Biodegradability Suggested in the Literature

Despite existence of works dedicated to building up QSAR models for biodegradability of organic chemicals, the comparison with the statistical characteristics of these models is limited because different authors are using different measures of the statistical quality. Only a few authors provide more or less standardized criteria. The model suggested in work [11] is characterized by R^2 = 0.96, but the authors have not provided the list of studied compounds. The best model suggested in [12] is characterized by R^2 = 0.951 for 17 polyaromatic hydrocarbons. Unfortunately, the predictive potential of the model is not checked up with external validation set. Classification models for biodegradability examined in [16] related to 200 organic compounds, but the comparison of the results from this work with models calculated with Eqs. 8–13 is impossible, because the authors have used the sensitivity, specificity, and accuracy as the measure of statistical quality of their models [16]. Model biodegradability for 15 anilines and phenols calculated with quantum mechanics descriptors [17] is characterized by R^2 = 0.955 and q^2 = 0.949. QSAR models suggested in work [3] are characterized by correlation coefficient for external validation set is ranged from 0.71 to 0.79. Thus, the statistical quality of models calculated with Eqs. 8–13 is quite good.

9 Conclusions

The CORAL software gives satisfactory predictive models for biodegradability. Molecular features, which are stable promoters of increase and decrease for biodegradability, are extracted (Table 8). The new molecular descriptors, which reflected presence/absence in the molecular structure five-member and six-member rings, improve the predictive potential of models for biodegradability for dataset 1 which contains acyclic molecules together with molecules that contain different rings.

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Glossary

QSAR	quantitative structure – activity relationships
CWs	correlation weights
SMILES	simplified molecular input-line entry system
CORAL	correlation and logic
RMSE	root-mean square error
R	correlation coefficient
q	leave-one-out cross-validated correlation coefficient
HSG	hydrogen suppressed graph

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Chapter 9

Biological Testing and Toxicity Bioassays in Biodegradation: Toward Better Process Control

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Abstract

The main purpose of bioremediation processes is to eliminate or even to reduce the risk of the toxic pollutants to reach safe and acceptable limits. Although environmental pollutants have complex nature and composition, most of the performed studies reported the application of methods for biodegradation of pollutants and xenobiotics in environment, especially wastewater, as a black box without considering sequestered risks. In this sense, chemical analysis alone is not sufficient for complete assessment of water quality while biological toxicity assays can estimate the effect of wastewater on the biota and assess the actual direct and sequestered environmental risks. This was the main driving force to teamwork in our lab to adopt, develop, and optimize biological testing methods and bioassays for better process control and real monitoring of the bioremediation efficiency. Several methods and bioassays were used such as BOD_5 and algal toxicity (to assess the degree of wastewater toxicity), *Artemia* toxicity (to assess impact on the aquatic creatures), phytotoxicity (to assess the impact on terrestrial plants). Also endocrine disruptors' assays and cytotoxicity (to assess the impact on mammalians and humans) were optimized. The authors discuss and transfer the knowledge and the step by step experience gained with these methods and bioassays. Moreover, this chapter elaborates all necessary practical tricks and precautions required to achieve accurate and reproducible measurements.

Key words *Artemia*, Phytotoxicity, Cytotoxicity, Microalgae, BOD, Toxicity, Endocrine disruptors, Pollutants, Biodegradation and bioanalysis

1 Biochemical Oxygen Demand Test (BOD_5)

1.1 Introduction

Among the commonly used bioassays in wastewater characterization is the BOD_5. Evidently, it is listed among the official tests in the standard methods for the examination of the water and wastewater [1]. The principle of the test is to measure the relative amount of dissolved molecular oxygen required for the biochemical aerobic degradation of the soluble organic matter in a sample of wastewater, effluent and polluted water at 23 ± 2 °C after incubation time of five days. BOD_5 easily provides a measure of the organic load in wastewater after incubation for 5 days. Hence, it is named BOD_5 test and is expressed as mg O_2/l. During this test,

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only partial degradation of the test organic matter occurs as complete degradation requires longer time (60–90 days). BOD_5 can also measure the nitrogenous demand; the amount of oxygen required to oxidize the reduced forms of nitrogen, so an inhibitor is added to prevent their oxidation [1]. The most interesting function to our teamwork was the use of BOD_5 in indirect measurement of the overall wastewater toxicity.

1.2 Materials

Special glass-sealed bottles.

Acclimatized microorganism (the seed).

50 mL glass flasks.

Rubber septa for sealing the flasks.

1.3 Methodology

In a study [2], the principle of BOD_5 was used to indirectly test the feasibility of microalgal-bacterial treatment of wastewater. An experiment was designed based on the BOD_5 test with different assembles (addition of microalgae and/or bacteria, or none of them [2]). The procedure for such an experiment was conducted as follows;

- Flasks of 50 mL were filled with 35 mL of wastewater samples/ artificial wastewater/culture medium supplemented with the pollutant and inoculated with 5% v/v of microalgal and/or bacterial inoculum.
- The flasks were then flushed with N_2 to remove atmospheric O_2 and sealed with rubber septa and agitated at 150 rpm at room temperature under continuous illumination (8000 lx) for 5 days (artificial wastewater) or 7 days (real wastewater).
- To monitor microbial respiration, gas samples of 150 mL were periodically withdrawn from the closed bottles using a syringe and injected on a GC (Agilent 6890N) equipped with a thermal conductivity detector (TCD). The carrier gas was helium at a flow rate of 30 mL/min and the columns used were a Hayesep N 80/100 9 ft. 1/8 in. and Molesieve 5 A 60/80, 6 ft. 1/8 in. The column, injector, and detector temperatures were 60, 105, and 150 °C, respectively.
- The correlation between the oxygen and carbon dioxide provided a direct measure of the suitability of the used consortia to further degrade the treated mixture; all experiments are carried out in duplicates or triplicates.

1.4 Notes

- The procedure of the BOD test and other experiments based on the BOD_5 principle are simple but may need some prerequisite analysis such as COD in order to estimate any necessary dilution to compromise the head air space and the challenged wastewater sample.

- The acclimated culture could be replaced by general microbiota such as activated sludge [1]. Therefore, whenever necessary, the diluted water sample is incubated with the acclimatized microorganism (the seed) in special glass sealed bottles at a specific temperature (23 ± 2 °C) for 5 days with agitation under darkness.
- This test will be performed at pH of 7.0–7.5, Therefore, buffer solution is to be added to keep pH constant or the samples pH are to be adjusted using suitable solutions at strength that will not dilute the sample by more than 0.5%.
- If the collected water samples will not undergo BOD_5 analysis within 2–6 h from collection, they must be stored at ≤4 °C.
- A negative control (glass bottle/flask filled with the diluted water sample without inoculation) should be prepared to avoid false BOD values.
- The acting microorganisms in the BOD_5 test could be either introduced by bio-stimulation through nourishing endogenous microbiota in the wastewater samples or by bio-augmentation through the addition of external activated sludge or even acclimatized consortia. The first strategy is more suitable in case of wastewater collected from domestic wastewater, unchlorinated or non-disinfected effluents from biological wastewater treatment plants, and surface waters receiving wastewater discharges. The second strategy (bio-augmentation) is commonly used if the sample is expected to contain a low level of microorganisms, a seed should be added as in case of the untreated industrial wastes, disinfected wastes, high-temperature wastes, wastes of pH values of 6–8 or wastes stored more than 6 h after collection.
- In case of the unavailability of seed adapted to degrade the wastewater, it has to be developed in the laboratory by continuously adding daily increments of the test to a soil suspension, activated sludge, or a commercial seed preparation under aerobic conditions. Whenever necessary, extra nutrient solutions have to be added to ensure that the microbial growth is not limited.
- Yet, the BOD_5 test suffers from some limitations:
  - Interference may occur due to nitrification denitrification processes.
  - Its considerable time length (5 days) and that the used inoculum may be affected by the fluctuation and toxicity of the tested water influent.

The second limitation point has been adopted by our team work to develop an interesting bioassay to monitor the feasibility of the subsequent algal-bacterial combination in wastewater treatment [2] or to monitor the efficacy of the photocatalytic and

photochemical pretreatment and see the possibility of the subsequent biological treatment of the pretreated wastewater [2, 3].

In a study, conducted by some of the team [3], the same principle was used to monitor the efficiency of the photocatalytic pretreatment of a mixture of phenol and *p*-nitrophenol where the treated mixture was tested for the subsequent biodegradation treatment using an acclimated consortia (AC) composed of a phenol-degrading *Alcaligenes* sp. and a PNP-degrading *Arthrobacter* sp., and microalga *Chlorella vulgaris* in comparison with the use of activated sludge (AS) as inoculum. In this study, glass flasks of 35 mL were filled with 25 mL of irradiated mixture and inoculated with 1 mL *C. vulgaris* culture and either 1 mL of AS or 1 mL of acclimated consortia. The flasks were then flushed with N_2 gas to remove any atmospheric O_2, sealed with rubber septa and aluminum caps and incubated for 5 days extended to 14 days under continuous agitation (150 rpm) and illumination (18 mW cm^2, Philips TLD 36 W/840 lamp). Controls consisted of flasks supplied with a pretreated mixture, inoculated (with each consortia) and incubated in the dark as well as flasks supplied with a non-irradiated mixture, inoculated and continuously illuminated. To monitor microbial respiration, gas samples of 150 mL were periodically withdrawn from the closed bottles using a syringe and injected on a GC as mentioned above. The correlation between the oxygen and carbon dioxide provided a direct measure of the suitability of the used consortia to further degrade the treated mixture.

In all inoculated flasks, O_2 was consumed and CO_2 was released during the first 4 days of incubation. This was likely due to microbial consumption of the dissolved O_2 initially introduced in the flasks (as neither the AS nor the acclimated culture was inhibited by the mixture), this is a typical BOD_5 based toxicity assay. Our team work further adapted this BOD_5-based assay and tested the effect on individual components of the microbial consortium by extending the incubation period to 14 days. Where the microbial activity was stopped in the flasks supplied with the non-irradiated mixture and illuminated as well as in the flasks supplied with the irradiated mixture and incubated in the dark. This was caused by O_2 depletion due to microalgal inhibition in the non-irradiated mixture or lack of photosynthetic activity in the pretreated mixture. In comparison, the levels of O_2 and CO_2 started to increase and decrease, respectively, in the illuminated flasks supplied with the pretreated mixture as results of photosynthesis. After 10 days of incubation, the O_2 level remained constant whereas CO_2 was undetected. This indicated that microbial activity has stopped due to the complete removal of biodegradable pollutants. This test has been successfully and extensively used by our team work in several studies.

2 Microalgal Toxicity Testing

2.1 Introduction

Microalgae are attractive photosynthetic phytoplanktons for eco-toxicity screening due to their sensitivity to various pollutants, their ease of cultivation and maintenance, and the small-volume samples they require [4]. Microalgae are a key element and rate limiting step in wastewater treatment methods based on microalgal-bacterial consortia because of their ability to remove N and P, produce the CO_2 (through photosynthesis) necessary for degradation by the bacteria, they can be harvested after the treatment process and valorized for use in some applications like biofuel production, as animal feed, bio-alcohol and biogas production and for plant fertilization [5–7].

Although different standard microalgal toxicity tests recommended the use of *Pseudokirchneriella subcapitata* microalga (formerly known as *Selenastrum capricornutum*), *Scenedesmus subspicatus*, and *Chlorella vulgaris* at certain cell density (1×10^4 cells/mL) under various experimental conditions [8], our team has adopted and optimized a microalgal toxicity assay using an identified *Chlorella* sp., this is due to its reported tolerance and resistance to various pollutants [6, 7, 9] and because it is the predominant genus to be found in wastewater treatment plants [3].

2.2 Materials

12 mL-Screw capped glass test tubes.

A purified microalgal strain (in the logarithmic phase of growth).

Mineral salt medium (MSM) or any other culture medium that can support the growth of the test algal strain.

LED or fluorescent lamps to provide illumination for microalgal photosynthesis.

Phosphate-buffered saline.

Pure methanol.

10 or 5 mL glass pipettes.

15 mL centrifuge tubes.

Spectrophotometer.

2.3 Methodology

Serial dilutions of the test pollutant or the wastewater samples were prepared in MSM supplemented with 2000 mg/l $NaHCO_3$; 10 mL of the prepared dilutions were transferred to screw capped glass test tubes of 12 mL.

- The chlorophyll-a content of the test algal strain (in the logarithmic phase of growth) is initially adjusted to reach 8–10 mg/l, Chlorophyll-a content is measured spectrophotometrically according to the method described by Chen et al. [10].

Fig. 1 Microalgal toxicity assay using *Chlorella* sp. performed in screw capped glass tubes

- All the tubes were inoculated with 5% v/v of the chlorophyll a-adjusted test algal strain, the tubes were then flushed with N_2 gas. Then the tubes were sealed with plastic screw caps.
- All the tubes were incubated under continuous agitation (150 rpm) and illumination (4000–5000 lx using either LED or fluorescent lamps), for 72 h.
- Controls were conducted under the same conditions but without adding the test pollutant or the wastewater sample. The test is performed in triplicates (Fig. 1).
- A volume of 5 mL samples was withdrawn and analyzed to measure the chlorophyll-a content according to the method described by Chen et al. [10].
- Microalgal biomass inhibition (%) was represented by the reduction of the average chlorophyll-a content in the test samples compared to that in the controls using the following equation:

$$\text{Chlorophyll a content}\left(\text{mg l}^{-1}\right) = 13.9 \times (\text{Abs}_{665} - \text{Abs}_{750})$$

2.4 Notes

- This test can measure the IC_{50} of chemical compound or pollutant by the determination of the concentration required to inhibit the growth of the test microalga by 50%, and so a comparison between a series of pollutants can be performed.
- An incubation period for 72 or 96 h will determine the inhibitory effect of the test pollutant or the wastewater sample on the growth of the microalga occurred during this period (IC_{72} or IC_{96}, respectively).
- There are some precautions to be in consideration while performing this test which include:
  - The source and concentration of the CO_2 source should be adjusted to be sufficient to support and allow selectively the algal growth and avoid any bacterial growth. Therefore, the

application of $NaHCO_3$ or other equivalents were recommended.

- The agitation rate of the test tubes should be adjusted to be sufficient for proper mixing and avoid any settlement of the algae on the walls of test tubes.
- In case of testing the inhibitory effect or toxicity of a pollutant liable to photodegradation by UV light emitted by the fluorescent lamps, LED lamps are recommended to use as the light source because they do not emit UV light.
- The screw capped tubes have to be sealed well to avoid leakage of the contents due to agitation during testing.
- The purity of the microalga has to be checked before the test to avoid possible contamination with another genus or bacteria that could have the ability to degrade the pollutant under testing.
- The air space has to be kept at minimum (low O_2 concentration) to avoid possible degradation of the test pollutant by the bacteria that may be associated with the test microalga and if necessary, the tubes have to be flushed with N_2.

3 Brine Shrimp (*Artemia* sp.) Toxicity Assays

3.1 Introduction

Artemia sp. (brine shrimp), a zooplankton, is an invertebrate component of the marine ecosystems that has been used for the assessment of general toxicity in teratology screens and in ecotoxicology [11]. Although ecotoxicological assays include those assays based on the growth inhibition of algae or plants, the mortality of crustaceans (shrimp) and their mobility inhibition are also employed in the assessment of wastewater and treated effluents toxicity [12]. They have been used previously for the detection of various types of pollutants as pesticides [13], heavy metals [14], and cyanobacteria toxins [15]. Moreover, the brine shrimp toxicity assays were employed in the assessment of toxicity of wastewater [12]. The toxicity of any substance that can be dissolved or dispersed in water can be studied using this bioassay. However, when dealing with liquid substance or wastewater, the assay is performed using several dilutions with ASW.

There are two commonly used brine shrimp toxicity assays; brine shrimp lethality assay (BSLA), based on the mortality of brine shrimp (*Artemia* spp.) and brine shrimp hatchability inhibition assay which is based on the inhibition of hatching of the cysts [16]. Our team has adopted and optimized the brine shrimp lethality assay for ecotoxicology assessments [7].

3.2 Materials

Cysts of *Artemia* sp.

Artificial sea water (ASW).

12-well plates.

Magnifier to aid in visuality of the nauplii.

3.3 Methodology

3.3.1 Brine Shrimp Lethality Assay (BSLA)

- The cysts are allowed to hatch in ASW (2 g cysts per liter) at temperature of 30 ± 2 °C, under continuous illumination of light intensity of at least 1000 lx and strong aeration conditions for 48 h in the hatcher [16, 17].
- The freshly hatched phototactic nauplii (in the instar II and/or III stages) are collected with a pipette in a small container.
- Different concentrations of the test substance are prepared in ASW such that the total volume per each well is 5 mL.
- Ten nauplii were carefully transferred into each well.
- Negative controls are prepared by adding 5 mL ASW and 10 nauplii without the test substance.
- The assay was performed in triplicates and the microplates were incubated at 30 ± 2 °C under continuous illumination of 1000 lx intensity for 24 h.
- The mortality of the nauplii was then observed and the % lethality was determined according to the equation:

$$\%\text{Lethality} = \frac{\text{No.of dead } \textit{Artemia} \text{ nauplii} \times 100}{\text{Initial no. of } \textit{Artemia} \text{ nauplii}}$$

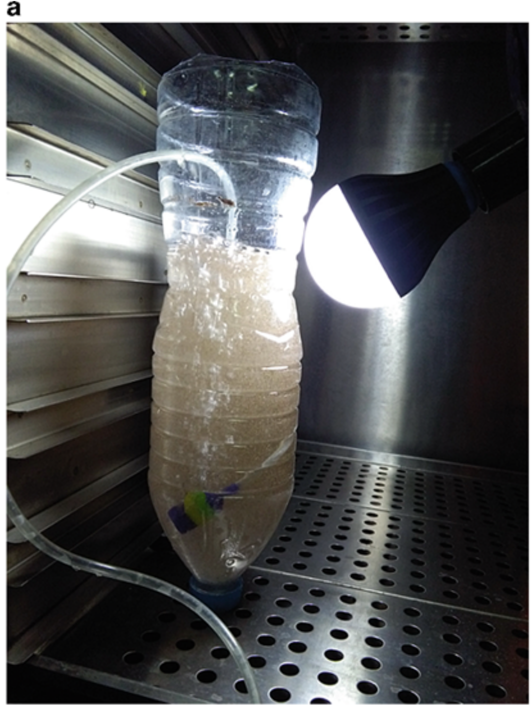
3.3.2 Brine Shrimp Hatchability Inhibition Test

Brine shrimp hatchability test is another toxicity assay that is performed using *Artemia* sp., it is based on the measurement of the dry weight of the nauplii freshly hatched in presence of different concentration of the test substance;

- One gram of the cysts is allowed to hatch as mentioned under BSLA in the presence of different concentrations of the tested substance.
- The newly hatched nauplii from 1 g of cysts are harvested on a membrane filter with pore size of 0.45 μm.
- The membrane is then placed in a desiccator at 60 °C for 24 h to obtain the dry weight [16].

3.4 Notes

- The dormant cysts are readily available at local aquarium stores at very low cost; It can survive up to 4 years if stored in a dry and cool place (refrigerator) and can be hatched easily using a simple equipment (Fig. 2).



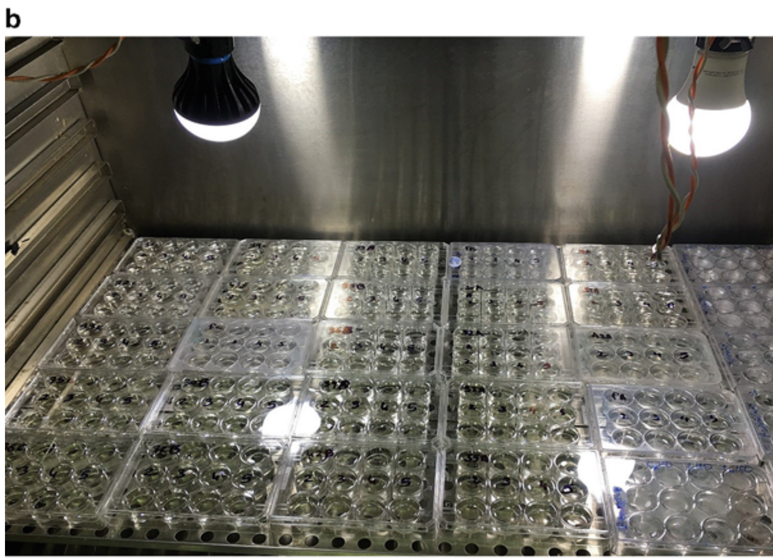


Fig. 2 (**a**) The container used for hatching the cysts of *Artemia* sp., (**b**) The brine shrimp lethality assay performed in 12-well microplates

- Freshly hatched nauplii can survive for up to 48 h without food because they still feed on their yolk sac [16].
- The freshly hatched nauplii are used while in instar II and/or III stages, during which, the nauplii are very sensitive to the external pollutants as their digestive tract epithelium is in contact with the outer environment [18].

- To avoid false positive results and to ensure that the mortality observed in the test wells is attributed to toxicity of the tested substance and not to starvation, the number of the dead nauplii in each sample is compared to the number of dead nauplii in the control.
- Nauplii were considered dead if their appendages did not exhibit any movement within 10 seconds of observation.
- In cases where control deaths were detected, the percentage of mortality (M%) was calculated as: M% = percentage of survival in the control - percentage of survival in the sample.
- Although brine shrimp toxicity assays are widely used in toxicity studies because of their ease of hatching from their dry cysts, yet important precautions should be considered regarding hatching of the cysts and the stage of nauplii to be used for testing [18, 19].
  - Hatching should be carried out under strictly controlled temperature conditions, as temperature was reported to affect the onset of hatching and the length of the hatching period [18].
  - On the other hand, the assay should be carried out with populations of nauplii at the same stage of development in order to get reproducible data. Since it was reported that different stages of the nauplii would make experimental data obtained more liable to fluctuation and not reproducible [18, 19].

4 Phytotoxicity Testing

4.1 Introduction

Plants seeds are resistant to harsh environmental conditions and can be stored for a long time. However, they are very sensitive to stress during germination and are therefore suitable for toxicity measurements [20]. Phytotoxicity assays are inexpensive, easy to perform, and suitable for aqueous, soil, and colored samples [20, 21]. Besides, phytotoxicity data for aquatic plants have served a relatively minor role in regulatory decisions concerning the environmental hazard of most potential contaminants. Aquatic plants were once considered to be less sensitive than invertebrates and fish to most toxicants. However, comparative toxicity data have proven this assumption to be incorrect in many cases.

A variety of phytotoxicity tests with freshwater green algae, duckweed, blue-green algae and diatoms, and rooted macrophytes (whole plants and seeds) have been described. Several test methods have been standardized for microalgae and are used primarily with chemicals and to a lesser extent with effluents, contaminated sediment elutriates, and hazardous waste leachates. Current scientific

understanding concerning the phytotoxic effects of these contaminants is based mostly on results for a few green algae. The greatest limitation of the test results is their uncertain environmental relevance due to the large interspecific variation in response of standard algal test species and the unrealistic experimental test conditions. Results of the few field validation toxicity tests conducted to resolve this uncertainty have been chemical-specific and non-predictable.

Plants are known for their sensitivity to pollutants and toxic substances which can interfere with their germination. Using plants as bioindicators for ecotoxicity measurement involves evaluation of the inhibitory effects on the early seed germination steps [22]. Therefore, the stem elongation inhibition test is used to assess the toxic potential of various contaminants, this also reflects the toxicity of low concentration soluble contaminants that are insufficient to inhibit the germination but can delay or inhibit the process of stem elongation [3, 23].

Some studies reported a reduction in the phytotoxicity values after the biodegradation of the pollutant phenol, *p*-nitrophenol, pyridine, and 2,4-dichlorophenoxyacetic acid [3, 9, 24]. Although different studies used different seeds for phytotoxicity testing like *Barbarea verna* (watercress), *Brassica oleracea* (kale), *Cucumis sativus* (cucumber), *Lactuca sativa* (garden lettuce), and *Rhaphanus sativus* (radish) [22, 25], our team has developed and optimized phytotoxicity test using *Lepidium sativum* seeds (cress) due to its high sensitivity to various pollutants and its availability throughout the year [26]. In the study performed by Tamer [20], *Lepidium sativum* and *Lactuca Sativa* were the most suitable seeds among 6 plants seeds to be used in phytotoxicity assays of toxic chemicals. However, *Lepidium sativum* showed better and more reproducible growth in this study and was therefore chosen as a test organism. The 3 day test duration was chosen for allowing germination rates >65% in blanks, according to the EPA test guidelines.

4.2 Materials

Seeds of *Lepidium sativum*.

5.5 cm (+) filter paper.

6 cm diameter glass pertidish.

Different concentrations of the toxic substance or the wastewater samples.

4.3 Methodology

- Different filtered dilutions of the samples are prepared, in case of wastewater samples, they are centrifuged and filtered prior to testing.
- 5.5 cm (+) filter paper is placed in each glass pertidish.
- 2 mL of sample dilution are added to the filter paper (pH 7).

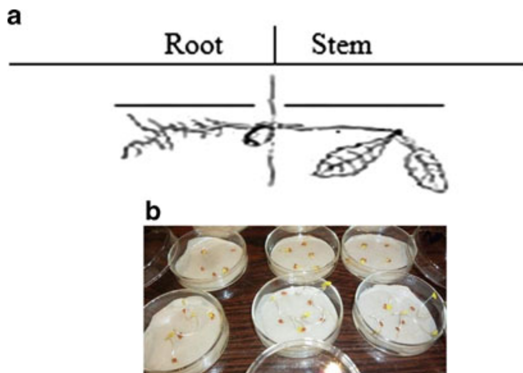


Fig. 3 (**a**) Vegetative parts of the germinating *Lepidium sativum* seeds, (**b**). Germination of *Lepidium sativum* seeds at the end of phytotoxicity testing

- Place five seeds of *Lepidium sativum* on the filter paper in each plate, the assay was performed in triplicates, i.e., 15 seeds were used for each sample/dilution.
- Petri dishes are incubated at 30 ° C for 5 day under complete darkness.
- Negative controls are prepared using the same vehicle for dissolving the pollutant or tap water (in case of testing wastewater toxicity).
- The average length of the stem is measured for both test and blank seeds (Fig. 3).
- Phytotoxicity % was calculated as the ratio of the reduction of average stem length of the test seeds to the average stem length of the control seeds.
- Potential outliers were identified and rejected by using the Grubb's test at the 5% significant level and results were analyzed with one-way ANOVA at 5% significance.

4.4 Notes

- Tap water is usually used as the vehicle to dissolve the toxic substance and preparation of the serial dilutions, and also in case of testing wastewater samples and used in case of the negative control.
- The incubation has to be performed under complete darkness to avoid the effect of photosynthetic activity of the seeds on its sensitivity to the test pollutants.
- The measurement of the toxic effect of the stem is much better and statistically applicable than the root.
- Checking the pH and the proportion of the sample to the size of the filter.
- Tap water is the best among other waters, for example, distilled water.

5 Endocrine Disruptors Assay

5.1 Introduction

Endocrine disruptors or endocrine disrupting chemicals (EDCs) are these substances which interfere with the endocrine system in particular. EDCs have been identified in terms of their anti/estrogenic, anti/androgenic, and anti/thyroid activities [27]. The presence of chemical substance with endocrine disrupting activity in water has been reported previously; for instance, reproductive abnormalities in alligators were reported as a consequence of insecticides spill in a lake in Florida, USA [28]. Chemicals with estrogenic activity have been detected in the effluents from wastewater treatment plants, this adversely affected the fish in the rivers receiving these effluents [29].

Recently, great attention has been dedicated to EDCs as a major water quality concern, it has been reported that EDCs with estrogenic activity have been significantly found in wastewater effluents, recent studies have utilized recombinant yeast strains to study the effect of EDCs on different hormone receptors [30].

5.2 Materials

Recombinant yeast (*Saccharomyces cerevisiae*) cells; yeast strain transformed with the estrogen receptor-α gene (ERα), estrogen-related receptor-γ gene (ERRγ), progesterone receptor gene (PR), or androgen receptor gene (AR). Yeast stably transfected with the tested gene and expression plasmids carrying a response element, while the reporter LacZ gene encoding the enzyme β-galactosidase is used.

96-well plates.

Z-buffer (16.1 g/l $Na_2HPO_4 \cdot 7H_2O$; 5.5 g/l $NaH_2PO_4.H_2O$; 0.75 g/l KCl; 0.246 g/l $MgSO_4 \cdot 7H_2O$).

Spectrophotometer.

5.3 Methodology

The yeast assay was conducted as described previously [31] as follows:

- Yeast strains are grown overnight at 30 °C, 130 rpm.
- The overnight yeast culture is then diluted with uncultured medium to reach an OD at 600 nm of 0.25.
- The tested sample is serially diluted in dimethyl sulfoxide (DMSO). Five microliter of the diluted sample is added to 955 μL of culture medium containing 5×10^3 yeast cells/mL; this mixture is called test culture.
- 200 μL of the test cultures are transferred into each well of a 96-well plate.
- The plate is incubated for 2 h at 30 °C, 130 rpm. The cell density of the culture is then measured at 600 nm.

- 50 μL of the test culture is transferred to a new 96-well plate, 120 μL of Z-buffer and 20 μL chloroform are added.
- The plate is vortexed for 25 s and incubated for 5 min at 30 °C.
- The enzymatic reaction is initiated by adding 40 μL O-nitrophenyl-*β*-D-galactopyranoside (NPG) (13.3 mmol/l, dissolved in Z-buffer).
- The plate is incubated on a titer plate shaker for 60 min at 30 °C.
- The enzymatic reaction is then terminated by adding 100 μL Na_2CO_3 (1 M).
- After centrifugation at 12,000 × *g* for 15 min, 200 μL supernatant is transferred into a new 96-well plate and the absorbance at 420 nm is measured.
- The *β*-galactosidase activity (*u*) is then calculated using the following equations:

$$u = \frac{C_S}{t \times V \times D \times \mathrm{ODS}} \quad C_S = \frac{10^{-6}(A_S - A_B)}{\varepsilon \times d}$$

where C_S is the concentration of the NPG in the enzyme assay reaction mixture.

t is the incubation duration of the enzymatic reaction.

V is the volume of the test culture.

D is dilution factor.

ODS is the OD at 600 nm of the test culture.

A_S is the OD at 420 nm of the enzyme supernatant of the sample.

A_B is the OD at 420 nm of the enzyme reaction supernatant of the blank.

ε is the extinction coefficient for NPG in the enzyme assay reaction mixture.

d is the diameter of the cuvette used.

5.4 Notes

- For each sample a minimum of five dilutions are prepared and samples must be assayed in at least triplicates. Each assay must include positive control (with substance has agonist effect) and a negative control (DMSO).
- It is important to measure the cell viability (spectrophotometrically at 600 nm) after exposure, to assure that any increase or decrease in the activity is due to agonistic or antagonistic response and not due to cytotoxicity.

The recombinant yeast assay was used previously to report estrogen-related receptor gamma (ERRγ) disruption activity in drinking water treatment plant located in north China [32]. Other studies used the recombinant yeast assay to illustrate the efficiency of three wastewater treatment plants (located in Spain) in eliminating the endocrine disruption activity by some estrogenic compounds [33].

6 Cytotoxicity Assay

6.1 Introduction

Biological assays represent an alternative methodology to assess the toxicity of complex water samples, as a global response to the complex mixture of chemicals is produced without any prior knowledge of the mixture composition or its chemical properties [34]. Cytotoxicity assays using different cell cultures (CHO, HepG2, Hu Tu 80) could be an alternative to assess biological toxicity of surface waters and may help to improve the control of water quality. Cytotoxicity assays are used to detect early damage, since water contaminants show two main effects on the cells: toxic effects (cell death) and proliferative effects which may be associated with carcinogenic effects [35]. Sulforhodamine B (SRB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assays are the most widely used methods for in vitro cytotoxicity screening [36–38].

6.2 Materials

Adherent cell line of interest.

Suitable culture medium.

Flat-bottom 96-well tissue culture plates.

Automated spectrophotometric plate reader.

6.3 Methodology

- Different dilutions of the tested substance/wastewater samples were dissolved in the culture medium and then filtered with a 0.22 μm membrane filter.
- Cell line was grown in tissue culture flasks maintained in the suitable culture medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/mL of penicillin, and 10 μg/mL of streptomycin.
- Only flasks containing fully grown monolayers were selected. The cells were then trypsinized (Trypsin 0.25%) and supplemented with fresh medium and 10% FBS.
- Cells were seeded in flat-bottom 96-well tissue culture plates in aliquots of 100 μL per well, and they were allowed to adhere to the bottom of the wells by growing in the sample-free medium for 18 h at 37 °C in a tissue culture incubator.
- After incubation, the growth medium was replaced with fresh medium (100 μL/well) in which different dilutions of the tested samples were prepared in step 1.
- One column of six wells was reserved for the negative control consisting of 100 μL of culture medium and 10% FBS.
- To prevent crossover contamination between the wells due to volatilization, a plastic adhesive sterile sheet was pressed over the wells before covering the microplate with a lid.

- The plates were then incubated for 72 h at 37 °C in a tissue culture incubator.
- After 24–72 h of exposure, the cytotoxicity could be measured by either the SRB methodology or tetrazolium (MTT) assays.

6.3.1 Sulforhodamine B (SRB) Assay

SRB cytotoxicity assay is adopted for a quantitative measurement of cell growth and viability, following the technique described by Skehan et al. [39] with minor modifications [40].

- Each well was gently aspirated, cells were then fixed by adding 100 μL of cold 10% (w/v) trichloroacetic acid (TCA), and incubating for 60 min at 4 °C.
- The plates were washed with distilled water (three times) and dried.
- 50 μL of SRB solution (0.4% w/v in 1% acetic acid) was added to each well, and incubated for 10 min at room temperature.
- Unbound SRB was removed by washing with 1% acetic acid.
- The plates were air-dried and bound stain was dissolved with 10 mM Tris base solution (150 μL/well).
- Optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 510 nm.
- The cell viability % and growth inhibition % were calculated using the following equation:

$$\text{Viability}\% = \frac{\text{mean OD sample}}{\text{mean OD control}} \times 100$$

$$\text{Growth inhibition}\% = 100 - \text{Viability}\%$$

6.3.2 MTT Assay

The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay can be used for the determination of cell viability and proliferation, as described by Mosmann [41] with minor modifications [38].

- Each well was gently aspirated, then 25 μL of MTT (5 mg/mL) solution was added to each well and the plates were incubated for 4 h at 37 °C. Living cells reductase enzymes reduced MTT to the insoluble formazan crystals that accumulate inside the cells.
- The medium was carefully aspirated and the wells were washed with phosphate-buffered saline and left to dry.
- 50 μL of dimethyl sulfoxide (DMSO) was added to each well, the microtiter plates were then left at room temperature for 30 min to dissolve the living cells and extract the formazan crystals.

- The MTT formazan product was determined spectrophotometrically using an automated spectrophotometric plate reader at 570 nm.
- The cell viability % and growth inhibition % were calculated using the same equations mentioned under the SRB cytotoxicity assay.

6.4 Notes

- The sulforhodamine B (SRB) assay depends on the ability of SRB to bind to the cellular proteins, so it does not differentiate living from dead cells [40]. While the MTT assay depends on cellular metabolic activity to reduce the colorless tetrazolium to the colored formazan, so it detects only living cells [40]. In previous studies, the SRB assay was compared to the MTT assay; it was reported that the MTT assay was less accurate in detecting changes in cell number as indicated by the variation observed in the linear range [36, 38]. Moreover, the MTT assay recorded interference with the tested glycolysis inhibitors (2-deoxyglucose, 3-bromopyruvate, and lonidamine) [38]. On the contrary, the SRB assay recorded the best results concerning all the tested parameters, with higher linearity with cell number, higher sensitivity and furthermore the cell staining was not cell line dependent [36, 38].
- Suggesting SRB assay as the most suitable and cost-effective assay for preliminary cytotoxicity screenings.
- The SRB assay was previously used for drug toxicity testing against different types of cell lines [42]. The cytotoxicity of wastewater, surface water, and potable water samples was assessed using MTT assay with human hepatoma (HepG2) cells [34, 35]. Again, the MTT assay was used as an efficient protocol to screen the suitability of drinking water without knowing the nature of the contamination, MTT assay tested the effect of microbes, and not chemical contaminants on the viability of intestinal human (Hu Tu 80) cells exposed to the tested water samples [43].

7 Biomass Settleability Testing

7.1 Introduction

Wastewater treatment using algal-bacterial consortia has been proven to be the most eco-friendly and cost effective too. Despite all advantages, a major drawback of algal based wastewater treatment ponds is the relative difficulty to remove the microalgal biomass from the treated effluent, a necessity to avoid secondary pollution [44, 45]. In this regard, the development of an assay to measure the settleability could offer a perfect filter for screening naturally auto-flocculating microalgal species. This has been

attempted by our teamwork and a settleability test was adopted, developed, and optimized [6] and this test was further applied in various studies conducted by the members of the current teamwork [9, 46].

Additionally, the biomass settleability testing was further developed and used as an indicator for the wastewater toxicity, compatibility of the microalgal-bacterial systems (flocs formation) and the efficacy and success of the treatment system [9, 47]. For instance, the microalgal settleability test was used to investigate the efficiency of a photobioreactor for treating artificial wastewater under various editions of $NaHCO_3$ and illumination durations [9].

7.2 Materials

100 mL glass measuring cylinders, 10 or 5 mL pipettes, 15 mL centrifuge tubes.

Wastewater samples containing the microalgal-bacterial biomass.

Phosphate-buffered saline and pure methanol for measurement of chlorophyll-a content.

Spectrophotometer.

7.3 Methodology

- The glass measuring cylinders were filled with 80 mL of the wastewater sample (Fig. 4).
- 5 mL sample were gently withdrawn from the uppermost layer (1 cm below the sample surface) at time intervals of 0, 10, 20, 30, 40, 50, and 60 min.
- Each withdrawn sample was subjected to analysis of the chlorophyll-a content according to the method described by Chen et al. [10] (as mentioned under microalgal toxicity testing).
- The settleability percentage of the biomass was calculated based on the reduction of the chlorophyll-a content at each time interval compared to chlorophyll-a content measured at time zero.

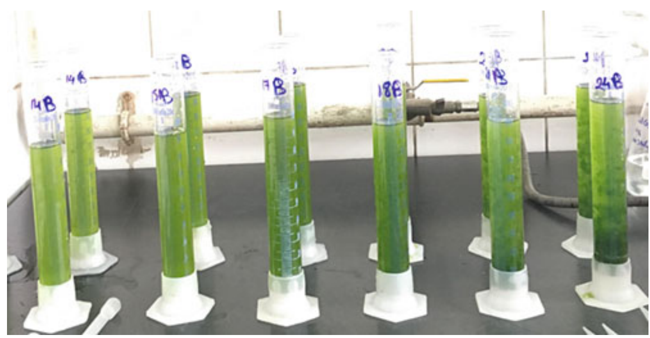


Fig. 4 Microalgal biomass settleability test performed in 100 mL measuring cylinders

7.4 Notes

There are some precautions when performing the biomass settleability testing:

- A good mixing has to take place to the sample before settleability analysis to ensure the biomass is still in suspension before testing to avoid false results.
- Pipetting and suctioning of the 5 mL sample at each time interval has to be performed very gently at the uppermost layer of the sample preferably at a depth not more than 1 cm from its surface, this prevents the disturbance of the settling process.
- There are some factors affecting the speed of the microalgal/bacterial biomass settleability, they include:
  - The presence of bacteria surrounding the microalgal phycosphere has an enhancing effect on the biomass settleability through increasing the floc-size of microalga. In case of axenic microalgal cultures, addition of flocculating agents such as Al^{+3} or Ca^{+2} salts leads to biomass flocculation; however, poorly settleable flocs are obtained in case no bacteria are associated with the microalgal cells [48, 49].
  - The pH of the culture medium affects the bioflocculation process, increasing the pH, as a result of CO_2 consumption due to microalgal photosynthesis or as a result of addition of alkaline salts such as NaOH, can neutralize the microalgal surface negative charge promoting the flocculation through increasing the particle size [47].
  - The ratio of the microalga: bacteria/activated sludge, a ratio of 5:1 proved to be the optimum to achieve rapid settleability within less than 30 min [47, 50].

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Chapter 10

An Overview of Methods to Detect Biodegradation Limiting Conditions

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Abstract

The chapter discusses some limitations to perform the successful results obtained with two methodologies widely used in laboratory scale to environmental conditions. Certainly, the microorganisms are the most important tools for the biodegradation process. For this reason, the biodegradability of any compound requires an active microbial consortium or an adequate succession of microorganisms. Also, the conditions to guarantee the microbial growth must be provided, such as micro and macronutrients, final electrons acceptor, optimal temperature, and range of suitable pH. We considered the temperature and pH as powerful factors to the biodegradation process, because the temperature defines the growth rate of the microorganisms and the pH plays a role in selecting the microorganisms per the different range of pH. In this chapter, we discussed how the natural biodegradation process, subject to dynamic environmental conditions such as temperature, pH range, and microbial succession, can possibly differ of experiments in controlled laboratory conditions.

Key words Respirometry, Colorimetry, Toxicity, Bioremediation

1 The Biodegradability and Environmental Conditions

The environment-friendly process based on biodegradation of toxic compound is the most important characteristic to bioremediation. In soil contaminations are many factors that might interfere in biodegradability of the contaminant. Contaminated fields require a characterization of the soil profile because it shows a heterogeneity composition in terms of size particles, organic matter, minerals, pH range, and others. However, in the laboratory scale, the sample is submitted to controlled conditions such as pH and temperature constant, homogeneity soil, and small amount of sample. However, the biodegradation of contaminated soil occurs in natural conditions, which are very dynamic and heterogeneous. Therefore, the

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challenge in biodegradation strategies is to bring the successful results of the laboratory scale to the environmental conditions.

There are many methodologies to determine the biodegradability of chemical compounds. The respirometric and colorimetric methods are widely used to measure the biodegradability of chemical substance. We discussed in this chapter about these two methodologies, especially about the controlled conditions under which they are performed, contrasting with environmental conditions in real scale.

2 Respirometric Methodology

2.1 Principles

The microbial respiration is a consolidated methodology to determine the biodegradability of chemical contaminants in soil as soil respiration is closely related to the microbial activity in the soil. The respirometric method allows quantifying CO_2 production and, consequently, estimating the biodegradation of the organic compound. Thus, any organic substance introduced into the medium can reflect on the microbial metabolism.

The Bartha and Pramer respirometer [1] is a closed system, consisting of two interconnected chambers as shown in Fig. 1. In a chamber, the material to be biodegraded is incubated and in the other one the KOH solution responsible for the capture of CO_2 produced by biodegradation.

The original method proposed by Bartha and Pramer [1] uses the quantification of carbon dioxide (CO_2) that reacts with the potassium hydroxide (KOH) to form potassium carbonate (K_2CO_3) as in Eq. 1. The quantification of CO_2 is measured by titration.

$$2KOH + 1CO_2 \rightarrow 1K_2CO_3 + 1H_2O \quad (1)$$

The other method possible to quantify the CO_2 production of microbial activity is through conductivity [2, 3]. This methodology is based on the releasing ions capable of changing the conducting electric current. Thus, the more CO_2 available to react with KOH, the more amount of K_2CO_3 will be formed, which will consequently reduce the solution conductivity. The linear correlation between the amount of K_2CO_3 produced and the change in conductivity is established. Based on the stoichiometric balance, the formed CO_2 could be determined accurately.

The respirometer apparatus developed recently is capable of measuring the microbial activity by Biological Oxygen Demand (BOD). The manometric respirometer records the oxygen uptake by the pressure drops in the closed system, which is proportional to BOD values [4, 5].

The respirometric method based on the measure of CO_2 produced is inappropriate in soil with high carbonate concentration due to

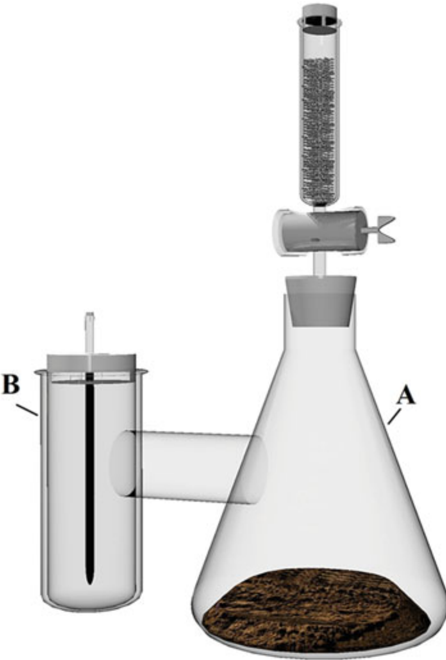

Fig. 1 Schematic drawing of Bartha and Pramer respirometer. The sample is incubated in chamber A and in chamber B is applied a solution of known concentration to capture CO_2 such as sodium hydroxide (NaOH) and potassium hydroxide (KOH)

2.2 Advantages and Restrictions

the release of abiotic CO_2. This release of abiotic CO_2 might be intense during the biodegradation, when the organic acids are formed, they react with the carbonate releasing CO_2. In a situation where it is impossible to measure the CO_2 production directly, it is possible to measure the respiration by the oxygen consumption.

The OECD [6] guidelines regards a substance as readily biodegradable when the theoretical production of CO_2 reaches 60% within a period of 10 days. The "readily biodegradable" means that the inoculum should not have been preadapted to biodegradation by previous exposure. A positive result for readily biodegradability can be considered indicative of rapid biodegradation in the environment. However, when the biodegradation has not been reached 60% of theoretical production of CO_2 until 28 days, the substance would not be classed as ready biodegradable [7]. There are many substances that need much more than 28 days to reach 60% of CO_2 production. The petroleum derivatives are a good example of very slow biodegradation. Because the different fractions of petroleum derivatives require a complex microbial community for the several catabolic pathways that lead to the mineralization [8–10].

2.3 Applications

The biodegradability test under laboratory conditions has the aim of obtaining a reliable draft of the biodegradation profile of contaminants in the environment. However, when the results from the respirometric method are submitted to seasonal variations, it can be affected by temperature, moisture, substrate, and minerals availability that support the microbial growth [11].

Under the environmental conditions the temperature is variable during the day, while in the biodegradation test the temperature is constant around the optimal temperature to microbial growth [12]. The study by Yadav et al. [13] simulated, in the laboratory, the natural seasonal change and diurnal fluctuations of soil-water temperature to investigate the impact on biodegradation. The authors concluded that both seasonal and diurnal fluctuations of soil-water temperature strongly influence the biodegradation time. The biodegradation time is increased almost twice more while changing from winter to spring/autumn and from spring/autumn to summer. The biodegradation increased twice for every 10 °C rise in soil-water temperature.

3 Colorimetric Methodology

3.1 Principles

The energy supply to microbial cell is redox reactions based on the transfer of electrons to synthesize ATP. Gillespie [14] suggested that bacteria exhibited reduction potentials when growing in the medium. Potter [15] had concluded that the break of organic molecule by microorganism resulted in liberation of electrical energy. The biodegradation process combines many redox reactions, in which carbon from the contaminants is incorporated into the microbial metabolism by the aerobic or anaerobic pathways.

In the aerobic pathway, the carbon is oxidized and the oxygen plays as the final electrons acceptor. In the anaerobic pathway, nitrate might be a final electrons acceptor, it is reduced to nitrite in a reaction catalyzed by the enzyme nitrate reductase. Other substances may act as final electrons acceptor such as iron, sulfur, ketones, manganese, among others. The many redox reactions in microbial cells are mediated by an important coenzyme, the nicotinamide adenine dinucleotide (NAD), in reduced form NADH.

The colorimetric tests are based on the capacity of an indicator to capture electrons from these redox reactions produced by microbial metabolism and energy source. This methodology has been recorded on the soil biodegradation because it is quick and inexpensive to detect microbial activity [16].

The 2,6-dichlorophenol indophenol (DCPIP) is a synthetic indicator of microbial activity [16–22]. This indicator shows a color blue in the oxidized form and is colorless in the reduced form. The accuracy method was tested by the studies of Kubota et al. [19]. The results indicate that the colorimetric test was

completely corresponded to chromatography analysis. Because samples that showed biodegradation by chromatography analysis also detected microbial activity in the DCPIP tests.

3.2 Applications

Cruz et al. [23] monitored the microbial activity of the diesel and biodiesel biodegradation using 2,6-dichlorophenol indophenol (DCPIP). The authors could observe that the faster reduction of the 2,6 DCPIP in the sample after incubation suggests natural selection of microorganisms that can metabolize the carbon source from diesel and biodiesel.

The 2,6 DCPIP are widely used for monitoring biodegradation test; however, Montagnolli et al. [22] proposed a novel point of view about redox reaction assays using 2,6 DCPIP as an indicator. The authors considered that the 2,6 DCPPIP discoloration is associated not only with substrate consumption, but it also discloses the whole microbial metabolism. For this reason, this study proposed a direct measure of toxicity of contaminated soil using redox reactions.

Also, the dehydrogenase activity has been used as measure for microbial activity of soil. Dehydrogenases are exclusively intracellular enzymes. The method is based on the use of triphenyltetrazolium chloride (TTC) or iodonitrotetrazolium chloride (INT) substrate as an electron acceptor. The TTC is reduced to triphenyl formazan (TPF) [24]. The dehydrogenase activity has been used in the biodegradation process as microbial activity index of soil [25–28].

3.3 Advantages and Restrictions

The neutral pH is the optimal pH range for dehydrogenase activity. For this reason, it is necessary to carry out the dehydrogenase test at neutral pH adding a buffering system because very low activity was found below the pH of 6.6 and above the pH of 9.5. The bacterial diversity was strongly related to soil pH [29]. However, when the dehydrogenase test is used to monitor the biodegradability of soil, the response of microorganism specialist of neutral pH will be favorable. The dehydrogenase test is not unique affected by the change of soil pH, but also the 2,6 DCPIP test. The biodegradation process of organic molecules trends to decrease the pH and the 2,6 DCPIP became red in an acid medium. In addition, the 2,6 DCPIP test is measured at 600 nm; however, in the acid medium, the absorption peak of 2,6 DCPIP was shifted from the 600 nm value [30].

The TCC reduction is inversely proportional to the soil aeration condition because the O_2 inhibits the TTC reduction [31]. Thus, if on one hand the O_2 inhibits TCC reduction, on the other hand, it is considered the most important molecule for microbial activities of aerobic microorganisms and very important on biodegradation. The first step to begin the biodegradation of alkanes is an oxidative process by incorporation of oxygen catalyzed by oxygenase and peroxidase [32].

We considered in this chapter the pH and the temperature two challenges to overcome in terms of biodegradation in environmental scale. Because both the factors have a huge impact on microbial growth. However, the biodegradation process might be affected by other factors, depending on the characteristics of the contaminated site.

4 Conclusions

The first step to a successful bioremediation is to bring accurate results about the biodegradability from the laboratory to the environment. The respirometric and colorimetric methods used to test the biodegradability of chemicals have some limitations when they are applied in the environment, because these methodologies are carried out under controlled conditions. However, they are methodologies largely used because they are simple, inexpensive, fast, and reliable under laboratorial conditions. Even with the points discussed in this chapter, the biodegradability test in the laboratory is a step very important for any bioremediation study. Therefore, it is up to the researcher to identify, in specific circumstances, which environmental conditions are limitation and to define how much it will affect the biodegradation.

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Chapter 11

Bioassays Used to Assess the Efficacy of Biodegradation

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Abstract

Many variables are involved in bioremediation process. Therefore, all biodegradation processes must undergo by a sequential tracking, in all steps, to identify and certify the efficacy of intended detoxification. The evaluation of effectiveness by bioremediation process of environmental samples has been performed mainly through chemical analysis, neglecting their effects on biological environment. In order to achieve a full evaluation and a consistent efficacy by the bioremediation process in all stages, besides the chemical analyses, bioassays are needed to estimate the real effects of pollutants and their metabolites over the biota. Thus, this chapter brings information about the main bioassays currently used to assess the efficiency of biodegradation/bioremediation applied to environmental contaminants, based on examples, endpoints and protocols.

Key words Bioremediation, Degrading organisms, Bacteria-based assays, Higher plants, Cultured mammal cell lines

1 Introduction

Bioremediation is considered a safe and efficient method to decontaminate polluted environments that could be applied in a broad variety of organic toxicants. This method uses living organisms that metabolize and degrade pollutants present in the environment, being considered with the most cost-benefit ratio [1]. However, many variables are involved in bioremediation, as the intrinsic biodegradability of each contaminant (e.g., recalcitrance), the appropriate growth substrate for each class of degrading organisms, the presence of microorganisms with enzymatic activity enough to degrade the target molecule, and the correct temperature to activate and maintain the degradation. So all biodegradation needs tracking in all steps, to certify its efficacy and to guarantee the absence of intermediary products that could be more toxic than the original pollutant [2].

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Effectiveness evaluation of environmental samples bioremediation could be performed only by chemical analyses, but this assessment estimates only the concentration of elements or toxic substances, not reflecting their real bioavailability [3]. Therefore, the association of chemical analyses with assays used to detect biological activity has been suggested in last years. Bioassays are recommended to monitor changes in the toxicity through bioremediation, mainly when contaminants are present as complex chemical mixtures [4, 5]. Thus, bioassays are designated to assess the stability of bioremediation regarding the toxicity [6], as they are capable of showing the bioavailability of sample contaminants, in each stage of bioremediation [7]. Another advantage of bioassays is the possibility of identifying additive, synergistic, and antagonistic effects exhibited by chemical components of a complex environmental sample [8]. In addition, they also permit the detection of toxicity shown by metabolites generated in biodegradation [9].

The choice of a correct biological assay to assess the toxicity of a sample is a challenging task, as each type of assay has different sensibility related to each toxicant and could measure better a specific endpoint [10]. Due to that, a battery of assays is the major recommendation to assure the complete bioremediation of contaminants, since these assays are based on organisms belonging to different taxonomic groups and diverse trophic levels, with various routes and exposure periods.

Although assays with eukaryotic organisms are considered more suitable to assess environmental toxicity, due to its proximity with humans, assays with prokaryotic organisms are the most routinely used, as they offer rapid results and show better cost-benefit. Plant assays are also effective to prove contaminants bioremediation, so they have been frequently used because of the ease of culture, low cost, and the possibility to test contaminants of different environment matrixes (e.g., soil, water, and air).

Among the assays based on prokaryotes, bacteria like *Escherichia coli*, *Salmonella typhimurium,* and *Vibrio fischeri* are the species more employed to test bioremediation effectiveness. Assays that use bacteria, like Microscreen and SOS-Chromotest (*E. coli*), Ames test (*S. typhimurium*), Mutatox®, and Microtox® (*V. fischeri*), are standardized for a long time and are accepted by international environment and health agencies, since they present good results. With the exception of SOS-Chromotest, Ames test, and Mutatox®, which detect mutagenicity, the others reveal the toxic potential of samples.

Regarding the plant assays, several species are used, from algae to higher plants, being these last ones the most frequently employed mainly based on seed germination index and growth inhibition (organism and roots length). Nevertheless, tests that have genetic parameters are considered more sensible for this class of applications.

In vitro assays with mammal cells are more expensive and require more rigorous details, as specific criteria for growth, mainly because of the need of an aseptic environment. Despite that, these tests show the advantage to have a good correlation with the effects observed in humans [11].

In this context and based on the scientific literature, this chapter intends to briefly describe some bioassays that have been satisfactorily used to prove the effectiveness of biodegradation/bioremediation of environment contaminants, as well as to provide examples of its applications to different classes of chemicals. Thus, this chapter aims to suggest a sequence of bioassays as a possible protocol to assist in biodegradation assessment, using test-systems that represent three different trophic levels (bacteria, plant, and mammal cell culture), with different grades of sensibility and endpoints.

2 Bioassays with Prokaryotic Organisms

Bioassays have been considered important tools to perform environmental risk assessment and, due to that, they have been frequently used in the monitoring of recovered environments through bioremediation [12]. Among the test-organisms used in these evaluations, prokaryotes deserve attention mainly because of its easiness to cultivate in laboratory, speed and effectiveness to detect biological activities of toxic metabolites generated during the biotransformation process.

Salmonella typhimurium assay, also known as Ames test or *Salmonella*/microsome, is considered a classic tool for assessing mutagenicity of several chemical agents [13], both for pure substances [14] and complex environmental matrices [15, 16].

Apart from being an assay used to register new medicines and chemical formulations [17], it also enables the detection of dangerous compounds in environmental samples, as those present in the water, air, soil, sediments, and effluent from various origins [18, 19].

This test is characterized as simple, quick and with high sensibility and reproducibility ratios [20]. In addition, the results obtained by the *Salmonella*/microsome assay present a high correlation with tests of rodent carcinogenicity, corresponding to 77–90% for positive responses [21], which makes it very attractive and reliable. According to Pohren et al. [22], this assay provides important data to characterize ecological risks of impacted environments, as well as to predict the possible effects arising from an organism exposure to environment pollutants.

The *Salmonella*/microsome test is performed with strains of *S. typhimurium* derived from parental LT2, auxotrophic for histidine (his-), which presents different mutation on this amino acid

operon, being built to detect mutations as frameshift or DNA pair base substitution. These strains are unable to grow in culture medium without histidine, unless mutations occur restoring their capacity to produce this amino acid. So, the number of revertants is easily measured by the counting of colonies that grows in minimal medium after the exposure of a cell population to a test substance [17].

Among the strains of *Salmonella* species available to perform the Ames test, TA 98 and TA 100 have been routinely used in assays of initial scan of xenobiotic effects, as they present more susceptibility to respond to a broad variety of chemical agents. The TA 98 strain detects frameshift mutations (caused by deletion or insertion of bases in transcriptable genetic material), while the TA 100 strain detects the damages induced by base-pair substitution. Other strains are also employed as TA 102 that is more sensible in the detection of agent inducers of cross-link DNA damages, while the strain TA 104 can be applied to prove the mutagenic effect related to DNA oxidative damages [17, 21]. Additional strains as YG 1041 and YG 1042 (derived from TA 98 and TA 100, respectively) are suitable to assess mutagenicity of samples with nitroaromatic compounds [23]. Regarding these properties, the choice of which strain must be used to perform an assay is mainly related to the chemical characteristics and mechanism of action of the tested agent [21]. Bioremediation assays require an in-depth knowledge about chemical properties. Evaluating the pollutant compound is important, as well as secondary metabolites that are yielded by biological processes. Such knowledge is important to choose a strain that responds satisfactorily to a given compound instead of resulting in false negatives.

The Ames test also allows the assessment of pro-mutagen compounds (substances that require biotransformation to exert a mutagenic effect) by means of the incorporation of a mammal metabolizing system, known as S9 fraction. Consequently, it is possible to obtain similar responses to mammal tests and expand the test sensibility [24].

Another assay commonly used to verify the effectiveness of contaminant biodegradation is Microtox®, which is based on bioluminescence inhibition. This assay uses the luminescence marine bacteria *Vibrio fischeri* NRRL B 11177 (formerly known as *Photobacterium phosphoreum*) to detect the toxicity of pure substances, both organic and inorganic, and mixtures included in different environmental matrices, as water, effluent, sediment, soil, sewage sludge, pesticides, including solid samples [25–27]. According to Rafaey [28], besides the simplicity of this test, it presents the possibility of assessing a large number of samples in a short time as an advantage, since the time required for an exposure is only a few minutes (5–30 min). Additionally, this test showed a high correlation of sensibility with toxicity assays performed with other

aquatic organisms, as fishes (*Cyprino carpio*, *Danio rerio*, *Oncorhynchus mykiss*), crustaceans (*Daphnia* sp., *Artemia* sp.), algae (*Chlorella* sp.), and protozoan (*Tetrahymena pyriformis*), especially in studies involving chemical substances containing only one reactive group (e.g., alcohols, ketones, aromatic compounds, etc.). Although verified in minor significance, it was also observed a correlation between Microtox® and toxicity tests with rats and mice, mainly in experiments with intravenous administration [29, 30].

The test principle is based on the decreasing of bioluminescence shown by bacteria exposed to a certain sample as a result of toxic biological activity. Luminescence is produced as a result of a chemical reaction that occurs between the luciferase enzymes, naturally present in these bacteria, the reduced flavin aldehyde, and oxygen [31]. Bioluminescence can also be considered a direct response of cellular metabolism, since it is directly related to the flow of electrons in the transport chain [32].

Microtox® results are, in general, presented as EC_{50}, i.e., the concentration in which a decrease of 50% of emitted bioluminescence is observed. Such a concentration is obtained through a dose-response curve created by the measurement of light intensity in different dilutions of the same sample [33]. Thus when comparing the results of different tested samples, those with the lower values of EC_{50} are considered potentially toxic.

Although the toxicity bioassays with prokaryotes have been used for a long time to assess environment impacts, with the development of biodegradation/bioremediation technologies they started to present an additional role that is crucial to prove the effectiveness of the process. In sequence, some examples of Ames test and Microtox® application in studies of biodegradation/bioremediation of pollutants or contaminated environments will be presented.

Steliga et al. [34] have used these two tests (Ames and Microtox®) to monitor the toxicity of soils contaminated with petroleum hydrocarbons submitted to in situ bioremediation. For 3 years of treatment based on soil drainage and bacteria and fungi enrichment, the authors observed an increase in toxicity resulting from the formation of intermediary toxic compounds, besides the degradation of the main substances. Due to that, the using of bioassays allowed to infer the ideal time of bioremediation, in which neither toxic nor mutagenic effect (Microtox® and Ames test, respectively) was observed, showing the complete mineralization of contaminants.

Mazzeo et al. [35] performed the Kado test (a variation of Ames test) using the TA 98 and TA 100 strains of *S. typhimurium* in the presence or absence of S9 metabolizing fraction to evaluate the efficacy of natural attenuation of sewage sludge from an urban area in different times. The mutagenic effect was observed during

the first three periods of degradation (0, 2, and 6 months) for the TA 100 strain in the presence of S9 fraction. Only after 12 months of natural attenuation the mutagenic effect induced by the sewage sludge was eliminated. Such results allowed establishing a safe time to assure the bioremediation, also showing toxic contaminants as pro-mutagens inducers of base-pair substitutions.

The Ames test was also employed to verify the degradation of Acid Red 114 azodye by microorganisms isolated from a sludge produced by a textile industry. The bacteria *Enterococcus faecalis*, *Alcaligenes faecalis* e *Achromobacter nitridificans* were effective to promote dye decoloration and removal of mutagenic effects, as observed using the *Salmonella*/microsome test. However, significant genotoxic and mutagenic results were found for a human cell line (HepG2), which shows less sensibility for this prokaryote test in the face of this class of contaminants [36].

Bilal et al. [37] used ligninolytic enzymes produced by *Ganoderma lucidum* fungi to biodegrade effluents from textile industries. In addition to an improvement in physicochemical parameters, the authors described a significant decrease in the effluent toxic potential. Among the bioassays used, it was possible to observe the decreasing of mutagenicity after biodegradation by Ames test (TA 98 and TA 100 strains), proving the effectiveness of the process.

Assays involving prokaryotic organisms have also been used to evaluate the biodegradation of micropollutants. Marco-Urrea et al. [38] have used the Microtox® test to assess the degradation of three medicines most used by general population (ibuprofen, carbamazepine, and clofibric acid). The authors concluded that, among the four species of white-rot fungi used in the experiment, only *Trametes versicolor* showed as promising to degrade these medicines. In addition, these same authors have observed through the Microtox® test that a metabolite even more toxic than ibuprofen was formed during the initial stages of its biodegradation. This result highlights the importance of bioassays to assure the efficacy of biodegradation.

Bonnet et al. [26] evaluated the toxic potential of different herbicides and some of their main metabolites (alachlor; diuron, and its metabolites D1, D2, D3, DCPMU, and DCPU; glyphosate and its metabolite aminomethyl phosphonic acid—AMPA) by the Microtox® test. The authors observed that, besides the chemical difference among these herbicides groups, all of them showed some toxicity. Regarding their metabolites, those produced by diuron biodegradation showed an even higher toxic effect. However, related to glyphosate degradation, AMPA showed lower levels of toxicity. Thereby, this study emphasizes the need to understand the mode of action of the main compound to avoid major environmental risk upon partial degradation of these herbicides.

3 Bioassays with Higher Plants

Plants constitute an essential part in ecosystems, thus they are also susceptible to environmental stress [39, 40]. Gopalan [41] stated that bioassays performed using this group of organisms could provide important information about the risk of environmental contaminants for different ecosystems, as aquatic, edaphic, and atmospheric.

Bioassays based on higher plants had already been established as genetic models to evaluate the effects of pollutants, being these assays widely applied in environmental quality assessment [42] and environmental monitoring [43]. US Environmental Protection Agency (USEPA) and World Health Organization (WHO) had validated these tests as efficient models to detect environment genotoxicity [44], while their protocols were standardized by the International Program on Plant Bioassays (IPPB) [39].

Besides genetic toxicity evaluation, plant bioassays can involve germination and growth tests. Assays that use seed germination ratio identify phytotoxicity of pure substances or complex mixtures, while those that use growth measurement evaluate changes in the stability and/or maturity of organisms as a result of an exposure to a toxic substance [45]. Genetic toxic evaluation in plants is the most used tool for monitoring the environment that shows details and suggests mechanisms of action, as these plant bioassays are based on the revealing of chromosomal aberrations, sister-chromatid exchange, and DNA chain breakages induced by xenobiotic [46]. Plants still present high sensibility related to various genetic parameters in different organs or tissues (roots, leaves, and pollen) [40].

Using plant organisms as environmental bioindicators brings many advantages when compared to other test-systems, as easy handling [42, 47], low cost, high sensibility, and efficient response to chemical agents, good correlations to other bioassays [47] including in vivo tests [48], and good applicability under different conditions of pH and temperature without results of false positive [40, 42]. Another benefit is related to exposure, which can be directly on test sample (pure or mixture of substances) without the need to perform procedures with the sample, as extraction or pretreatments [48].

Due to these features as bioindicators to assess pollution, this group of organisms also contemplates the characteristics as great test-systems to monitor biodegradation process [9, 49–51].

Among plant species, the most commonly used to assess environmental contaminants are *Allium cepa*, *Arabidopsis thaliana*, *Capillaris crepis*, *Glycine max*, *Hordeum vulgare*, *Lactuca sativa*, *Nicotiana tabacum*, *Tradescantia* sp., *Vicia faba*, and *Zea mays* [40, 44].

Allium cepa test is one of the most used in environmental assessment, because it allows the evaluation of different genetic mechanisms, as: germination index, related to organism toxicity; mitotic index, related to cytotoxicity; chromosomal aberration index, related to contaminant induced-alterations in structure and number of chromosomes of the cells; nuclear abnormalities index, related to morphological alterations in interphase nuclei as result of a test substance; micronuclei index [43], related to the induction of extra nuclear bodies containing chromatin material derived from chromosomal breakages or aneuploidy [46]. Both chromosomal aberrations and micronuclei are described in the scientific literature as efficient indicators of direct action of a substance on the DNA molecule [43]. Thereby, chromosomal aberrations and nuclear abnormalities are considered markers of genotoxicity, while micronuclei are considered markers of mutagenicity.

Srivastava et al. [52] performed a bioremediation by vermicomposting with an urban sludge collected in a landfill near an automotive industry in Uttar Pradesh (India) for 30 days. The authors evaluated the sludge chemical composition and monitored the phytotoxicity and genotoxicity of the samples by *Allium cepa* assay. Aqueous extract of the samples was prepared and applied in the experiments. After vermicomposting, it was observed a quantitative decrease in all metals present in the sample (Cr, Cu, Ni, and Pb). Roots of *A. cepa* exposed to nontreated samples showed significant frequencies of chromosomal aberrations, while degraded samples did not induce damages. With these results, the authors concluded that this bioremediation was effective to detox the sludge, as well as the test-organism was efficient to monitor the entire process.

Mazzeo et al. [51] performed the monitored natural attenuation of sewage sludge from an urban area of Rio Claro, located in São Paulo state, Brazil, with the aim of assessing its possible decontamination after several periods (0, 2, 6, and 12 months). The experiments were done with pure sludge and with soil associations, in different proportions (10, 25, 50% of sludge). Chemical analyses and experiments with *A. cepa* were used to assess the effectiveness of the process. As result of chemical analyses, *m*- and *p*-cresol were identified in concentrations higher than those determined by the Brazilian laws in both initial and 2 months degraded samples. This result was not found for the other samples collected in later periods of natural attenuation. Regarding biological assays, the authors verified the induction of damages in the genetic material of *A. cepa* by pure sewage sludge and its aqueous extract, even when associated with soil. However, it was observed a decrease in these effects proportionately over time of bioremediation, with the exception of the highest concentration (100% of sewage sludge). The authors concluded that crude sewage sludge is not a viable material to be used for restoring soil, even after natural attenuation.

However, they emphasized that this process is efficient only when the sludge is mixed with soil and lasting at least 12 months. The using of *A. cepa* as a test-organism in this assessment proved its applicability to measure detoxification of residues.

Mena et al. [53] applied the composting process with wood shavings with sewage sludge from treatment station, located at Murcia, Spain. The biodegradation efficiency was verified by means of germination assays with *Hordeum vulgare* and *Lepidium sativum*. These researchers observed inhibition of germination when seeds were submitted to sludge prior to biodegradation, whereas germination ratios were increasing according to composting periods, i.e., toxicity was decreasing while biodegradation was decontaminating sewage sludge. These authors related the decreasing of samples toxicity with possible organic matter stabilization.

Souza et al. [54] used *A. cepa* to assess biodegradation of petroleum hydrocarbons with landfarming, before and after adding rice hulls, aiming at chromosomal aberrations, micronuclei, and nuclear buds induction. Results showed that the initial sample had 13.5 g of hydrocarbons per kilo of landfarming material, inducing clastogenic and mutagenic effects. After 108 days of biodegradation, hydrocarbons concentration decreased as well as all types of damages were diminished. The adding of rice hulls provided greater decreases for hydrocarbons concentration and also for toxic effects. So the authors concluded that rice hulls had optimized the biodegradation process.

Hund and Traunspurger [4] prepared in situ biobed treatment in soil contaminated with polycyclic aromatic hydrocarbons (PAH). During the process, water and nutrients were added and the matter was revolved each 4 weeks to enhance oxygenation. In this work, chemical and ecotoxicological analyses were done. Ecotoxicological tests were performed based on the growth of *Brassica rapa* and *Avena sativa* exposed to samples collected in 0, 4, 7, and 11 months of biodegradation. Chemical analyses results revealed a decrease in PAH concentration in direct proportion to the increase of bioremediation time. With regard to ecotoxicological assay, samples collected in first 4 months of degradation had inhibited both plant growth, but after 7 and 11 months of bioremediation plant development was returned to normal. It was also possible to note *B. rapa* as more sensible than *A. sativa*. These data are linked to the information obtained through chemical analyses, because as the biodegradation occurred the concentration of PAH bioavailable was diminished, followed by toxicity decrease.

Phillips et al. [49] studied bioremediation applied to three samples of Canadian soil contaminated with creosote. Remediation monitoring was verified by different bioassays, including germination test with *Lactuca sativa*. These researchers observed that despite the decreasing in total concentration of hydrocarbons after the treatment, there was a significant increase in toxicity by

inhibition of germination. Probably, intermediary toxic metabolites generated during biodegradation were the responsible for this result, which shows the importance of associate chemical analyses with toxicological tests, as chemical studies solely are not enough to estimate biological impacts caused by a contaminant.

In another study, Mazzeo et al. [50] implemented the biodegradation of five distinct concentrations of BTEX (benzene, toluene, ethylbenzene, and xylene mixture) by pre-selected microorganisms. The authors evaluated the efficacy of the process with chemical and biological analyses, being these last by means of *A. cepa* test. Results showed that biodegradation decreased genotoxic and mutagenic damages induced on *A. cepa* test-organism. Thus, this study showed the efficacy of hydrocarbons biodegradation by pre-selected bacteria extracted from sediments removed from stabilization pond used by a petroleum industry as well as it highlighted the effectiveness of *A. cepa* as a good bioindicator to assess biodegradation.

Migid et al. [55] studied the toxicity of an industrial effluent contaminated with chemical fertilizers, located in the province of Dakahlia, Egypt. In addition, they evaluated the efficacy of its remediation by algae biofilters. Ecotoxicological tests were done with *Allium cepa* and *Vicia faba* to identify cytotoxicity and genotoxicity before and after the treatment with different algae biofilters. It was observed toxic effects only before bioremediation due to the decline of mitotic index and high frequencies of chromosomal and nuclear abnormalities in both species. After bioremediation with algae biofilters, there was a significant decreasing in cytotoxicity and genotoxicity related to depletion of heavy metals concentrations. Both species were considered good to monitor remediation of effluents.

Raj et al. [56] studied the bioremediation of effluent generated by a pulp and paper industry (Saharanpur, India) by the bacterium *Paenibacillys* sp. isolated from the soil located at the same place of effluent discharge. Toxicity was analyzed by germination tests and root growth measurements with seeds of *Vigna radiata*. Seeds exposed to crude and non-diluted effluent did not germinate, while seeds exposed to diluted effluent (25 and 50%) without treatment have germinated, presenting higher growths exhibited by the sample less concentrated. As expected, seeds exposed to bioremediated samples showed higher roots growth when compared to those exposed to non-degraded effluent. These results suggest the reduction of toxicity after the remediation treatment with bacteria culture due to degradation of toxic compounds.

Ventura-Camargo et al. [57] evaluated the cytotoxic, genotoxic, and mutagenic effects of two concentrations (50 and 200 μg/L) of black dye commercial product (BDCP) used in textile industries in meristematic cells of *A. cepa*, before and after biodegradation by bacteria isolated from a wastewater treatment station.

Cytotoxic and genotoxic effects were detected in cells exposed to non-biodegraded or biodegraded samples. Genotoxic alterations were more frequently observed after exposure to biodegraded samples when compared to non-biodegraded ones, showing the generation of metabolites by bacteria that were more toxic than the origin compound. Based on these effects, the authors concluded that bacterial biodegradation is not always positive, because it can produce compounds that are more detrimental to the environment.

Chelinho et al. [58] did an experiment aiming to assess the biodegradation of herbicide atrazine involving bioaugmentation with *Psedomonas* sp. and biostimulation with citrate. Three doses of Atrazerba FL (commercial formulation of atrazine) were spiked to soil microcosms: the recommended dose (RD; 2 L/ha), 10× RD and 20× RD to simulate overuse/accidental spills scenario. Besides chemical analyses, ecotoxicological endpoints were assessed by measuring biomass production by *Avena sativa*. Non-bioremediated soils have induced an expressive decreasing in plant biomass when compared to the biomass found in correspondent bioremediated soils. Chemical analyses revealed faster degradation of atrazine in bioremediated soils than in nontreated samples. Therefore, the combination of bioaugmentation and biostimulation was an effective tool for reducing the toxicity of soils contaminated by atrazine.

A bioremediation study involving a solid residue, sugarcane filter cake (SCFC), derived from ethanol and sugar industry was done by the authors in [59]. These authors used *Allium cepa* test-organism to assess genotoxicologic effects, before and after 3 and 6 months of bioremediation applied to pure SCFC or with it associated with sugarcane bagasse (SCB) and soil. Both solid and aqueous extracts were evaluated. Chemical analyses revealed decline in metal content, total organic carbon, and nitrogen after 6 months of biodegradation. Before this process, it was observed cytotoxic and genotoxic effects for the tested samples, with the exception for the association of 75% of SCFC + SCB + soil. Samples that were bioremediated by 3 months showed higher cytotoxicity than initial samples, and also presented genotoxic and mutagenic effects. After 6 months of bioremediation, both solid and aqueous extracts of SCFC + SCB + soil association did not induce toxic effects in *A. cepa*, showing effectiveness by the bioremediation process. This study also proved the applicability of *A. cepa* test-system as capable of being used for monitoring cytotoxicity, genotoxicity, and mutagenicity of samples submitted through bioremediation.

Therefore, it is evident that many studies apply biological assays based on plants for monitoring the bioremediation process and that a joint use of plant bioassays with chemical analyses is fundamental for the real diagnostic of biodegradation.

4 Assays with Cell Culture

Toxic effects of contaminants can be decreased by means of biodegradation, whose adverse effects can and must be monitored through different bioassays. In addition to tests based on prokaryotic organisms and higher plants, as described previously, bioremediation efficacy can also be assessed by assays performed with cultured mammal cells.

Mammal cell culture has been used as test-system in many assessments of toxicity by environmental contaminants [60–63]. This tool is also used to identify the preservation of toxic effects by environmental pollutants after treatment with biodegradation and bioremediation processes [35, 60, 64–66], which could define the disposal or the safe reuse of certain residues.

Assays based on mammal cell culture bring important information about the mode of action of contaminants present in the environment [61, 63] as of controlled/isolated substances [62]. Cell cultures can be used to assess different endpoints, as cytotoxicity, genotoxicity, and mutagenicity, allowing a broad range of evaluations [60].

Cell viability tests are the most routinely used to assess cytotoxicity. Among these assays, MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) is rapid and quantitative [67], widely used in cell lines maintained in cultures to assess cytotoxicity of several substances [68]. This test is based on the ability of living cells to reduce MTT salt (Thiazolyl Blue Tetrazolium Bromide—a yellowish hydrosoluble substance) in formazan crystals (insoluble blue salt), which precipitates by the action of mitochondrial enzymes as succinate dehydrogenase. These crystals are insoluble and cannot cross the membrane of viable cells, accumulating inside [68].

Another method used to assess cytotoxicity in cultured cells is neutral red retention assay. Neutral red is a supravital dye used to evaluate the capacity of viable cells to incorporate and retain it by lysosomes when submitted to toxic substances [69]. Neutral red assay is a high precision in vitro test that identifies the cytotoxic potential of samples, determining the maximum non-toxic concentration. This dye can cross cell membrane by diffusion and can concentrate on lysosomes, where it binds to anionic and phosphate groups present in the lysosomal matrix. In contrast to MTT that must be metabolized, the entry of neutral red in the cell depends on the cell capacity to maintain a pH gradient by the ATP synthesis. In physiologic pH, the dye almost presents neutral charge, which allows its entry through cell membranes. Inside lysosomes there is a protons gradient that maintains lower pH than in cytoplasm. Thus, the dye turns charged and becomes retained in this organelle, while in dead or damaged cells this event stops. Finally, this test

allows the differentiation of viable from dead/damage cells according to the lysosomal capacity to retain the dye [70].

Besides assays to assess cytotoxicity in mammal culture cells, there are those designed to evaluate genotoxicity and mutagenicity of compounds, as well as to determine the possible decreasing of its effects after biodegradation. Comet assay is the most used genotoxicity test because it is considered quick, simple, and sensible to detect damages induced by alkylating, intercalating, and oxidative agents on the DNA molecule of individualized cells [71, 72]. According to Speit et al. [73], comet assay gained popularity for being a test capable of detecting low levels of DNA damage and repair activity, and also for being applicable for several types of cells and tissues. The alkaline variation of comet assay theoretically can be applied to any animal tissue both in vitro and in vivo, as long as the cells are isolated [74]. Another advantage of this assay is the need of a small amount of cells in any cell cycle stage [75].

The in vitro micronucleus test, also known as cytokinesis-block micronucleus assay, uses cytochalasin-B to suppress actin polymerization that is essential to form microfilaments related to cytoplasm contraction during cytokinesis [76, 77]. As this substance does not interfere in karyokinesis, bi-nucleated cells are formed from a division cycle [78, 79].

This assay also allows observing nuclear buds and nucleoplasmic bridges established between nuclei of bi-nucleated cells. According to Fenech [80] these last structures probably arise from dicentric chromosomes separation, when both centromeres are pulled to each cell pole. In addition, nuclear buds also can appear from extra genetic material, which can be expelled from the main nuclei. Thus, the presence of nucleoplasmic bridges and nuclear buds on bi-nucleated cells can also be considered genotoxicity evidence, which must be rated concomitantly during the analyses. The micronucleus test followed by these analyses is recognized as micronucleus cytome assay (CBMN-Cyt) [81]. Due to these features, this technique is frequently used to detect DNA damages caused by clastogenic and aneugenic agents [78, 82]. CBMN-Cyt is widely used with cultured cells applied to monitoring environmental impacts, because it evaluates genotoxic/mutagenic potential from agents present in the environment [79].

Ayed et al. [64] assessed the toxicity of two classes of industrial dyes (azo and triphenylmethanes) on epithelial human cell line (Hep-2) before and after biodegradation. By means of the MTT test, the authors determined the initial cytotoxicity of both dyes through the inhibition of cell viability (IC_{50}), in which azo dyes presented IC_{50} from 10 to 65 ppm and triphenylmethanes presented IC_{50} from 25 to 35 ppm. After biodegradation by

Staphylococcus epidermidis bacteria it was verified a significant decreasing in those compounds effects until non-toxic levels were reached [64].

Still approaching toxic effects and biodegradation potential for azo dyes, Bafana et al. [83] evaluated the potential to detoxify a known toxic and carcinogenic dye (Direct Red 28—DR28) by bacteria *Bacillus velezensis*. In the evaluation, these authors used specific and sensible cytotoxicity and genotoxicity assays, as comet assay and flow cytometry with HL-60 cells (human promyelotic leukemia cell line). This research results showed absence of toxic effects for initial samples, but after 3 days of biodegradation its metabolites were capable of inducing DNA damage and cell death, mainly by apoptosis. After 15 days of bacterial activity those toxic metabolites were also degraded and toxic effect ceased.

In another study, Das et al. [84] investigated the toxic potential of a paper mill effluent after biodegradation by *Pseudomonas* sp. strain ISTDF1 by assays based on human hepatocarcinoma cells (HuH-7). MTT and comet assay were used to evaluate the effluent cytotoxic and genotoxic effects respectively, before and after biodegradation. The MTT test showed a decrease for cytotoxic potential as bacteria biodegradation period increased and the same pattern was observed by the results obtained by comet assay concerning genotoxic potential.

As well as evaluated by Das et al. [84], Mishra et al. [85] also assessed the toxicity of paper mill black liquor before and after biodegradation by bacteria of *Bacillus* genus. These authors have created aerated soil microcosms containing the residue and bacteria. Biodegraded and non-biodegraded samples were collected after 2 and 15 days of exposure to microcosms. Such toxic as biodegradable potential was measured by MTT and comet assay with HuH-7 cells. Chemical analyses by gaseous chromatography showed a decline in concentration of all substances present in the residue before the treatment with microcosms. These changes could be the result of compounds transformation to simple substances with great potential to mineralize. The MTT test proved the efficacy of bacterial biodegradation as after 15 days of remediation was verified a 100-fold decrease of cytotoxicity when compared to crude samples. Regarding the genotoxic potential, after 15 days of treatment the residue presented tenfold less harmful. In addition, the authors quantified the presence of large amounts of reactive oxygen species (ROS) in cells exposed to the crude residue and a very small portion in cells exposed to remediated samples. Thereby, the authors considered bacterial bioremediation as feasible to detoxify this residue from paper mill and they highlighted the applicability of human cultured cells to assess its efficacy.

As pulp and paper industry, electroplating industry is also considered an important source of toxic residues. In this context, Naik et al. [86] used HuH-7 cells to estimate the biodegradation

effectiveness to detoxify an electroplating effluent, rich in hexavalent chromium. Metal removal by activated charcoal was compared to the process of bio-sorption provided by bacteria, fungi, yeasts, and agricultural products separately, and it was observed that the combination in sequence of activated charcoal and bacterial degradation was more efficient, with the removal of 99% of chromium (VI) present in the effluent. By means of the MTT test, the authors determined an expressive decline in cytotoxicity after the combined treatment. By comet assay results it was possible to observe a sixfold decreasing of effluent genotoxic potential when compared to results obtained by non-biodegraded samples. Thus, the authors concluded that the sequential physico-biological treatment could be a solution to mitigate cytotoxicity and genotoxicity induced by metal-rich effluents, as those carrying chromium (VI).

Petroleum derivatives constitute another class of contaminants that deserves attention about toxicity assessment. As explained previously, BTEX is a complex mixture of compounds with great potential to impact water resources, especially groundwater. Mazzeo et al. [60] applied biodegradation of BTEX with a bacterial pool obtained from a stabilization pond from a Brazilian petroleum industry. These authors assessed the detoxificant efficacy of BTEX after 20 days of bacterial biodegradation by means of micronucleus test and comet assay with hepatoma tissue cells (HTC). In general, it was verified the genotoxic potential of BTEX even in small concentrations and the mutagenic potential for those concentrations close to its components water solubilization. Bacterial biodegradation was effective as genotoxicity was decreased and mutagenicity remained absent after the processing, showing that the metabolites formed were not toxic to tested cells [60].

Similar to petroleum derivatives, insecticides are also classified as a worrying group of contaminants. Insecticides can act by several ways due to its physicochemical properties. Among them, endosulfan is a hydrophobic compound in nature that can get sorbed to soil and sediments turning it more persistent in the environment. Due to its recalcitrance to biodegradation, this compound is easily bioaccumulated and biomagnified though trophic web reaching non-target organisms, including humans [66]. Facing this concern, Sundaram et al. [87] studied the degradation of cypermethrin insecticide by *Bacillus* sp. in microcosms and also assessed its toxicity by in vitro tests with cultured human cells. These researchers have isolated degrading bacteria and, then, they have monitored the biodegradation process for 0, 5, 15, and 30 days through chemical analyses and cytotoxicity and genotoxicity tests with HuH-7 cells. The authors observed that bacteria from *Bacillus* genus were effective to degrade endosulfan and they registered the mechanism of metabolization used by them. The MTT test indicated a significant fall in cytotoxicity after 30 days of biodegradation as the LC_{50} was raised 10^5-fold when compared to the

insecticide cytotoxic potential without treatment, i.e., it was much less toxic. Regarding genotoxic potential, after 30 days of treatment the DNA damage ratio was reduced to values three times lower than those obtained for the insecticide in the pure form. By these results the authors concluded that the microbial consortia was successfully applied to decrease cytotoxic and genotoxic potential of cypermethrin, besides classifying HuH-7 cell line as sensible in this nature of assessment.

When studying biodegradation of endosulfan, Kumari et al. [66] used the degrading strains of *Paenibacillus* sp. ISTP10 and tested its yield with tests done with human hepatoma cells (HepG2 cell line). After isolating these bacteria characterized as endosulfan degrading, several microcosms were set up with endosulfan samples for 0, 48, 120, 360, and 720 h of biodegradation. The authors concluded based on the results obtained through MTT test that endosulfan was better degraded and had its cytotoxic potential reduced starting after 48 h of bacterial activity, but with best results after 720 h. Concerning genotoxic potential, the authors observed a drastic decline in DNA damage by comet assay in a similar way as evidenced for MTT. These results showed that endosulfan was satisfactorily degraded and, consequently, a significant decreasing of its toxic effect was related to the degrading action of bacteria *Paenobacillus* sp. These data also confirmed that biodegradation processes using this microorganism show more effectiveness after 30 days of activity (720 h).

Aside from chemical compounds coming from industry or agriculture, urban rejects also become a concern when the context is environmental impact. With pronounced urban development and increasing population sizes in cities, sewage treatment has turned a challenging factor for treatment stations that generate large amounts of sludge. Mazzeo et al. [35] monitored the possibility to naturally attenuate the sewage sludge toxicity to reuse it as a reconditioner of agricultural soils. In this regard, the authors have applied micronucleus test with HepG2 cells to assess sewage sludge toxicity before and after 2, 6, and 12 months of biodegradation. When this process was performed for 6 and 12 months it was shown as effective for reducing genetic damages in human cells when compared to non-treated samples [35].

Nowadays, the risk of contamination threats environmental health in such a way that researchers were directed to evaluate compounds that were neglected in the past. So when we talk about pollutants or contaminants of the present, emergent contaminants have gained attention from the scientific community and from environmental agencies. Among the compounds included in this class of contaminants are the pharmaceutical products. These chemicals, which are related to high human and veterinary consumption, deserve great attention, mainly regarding its effects on biological environment. Among these contaminants are antibiotics,

which have been attracting researchers to search new methods to diminish their environmental impacts. In this context, Krifa et al. [65] have studied the ability of *Pseudomonas putida* to grow in effluent containing antibiotics as amoxicillin and cefadroxil and to degrade these compounds. After achieving successful growth of bacteria, the authors evaluated the effluent cytotoxicity with MTT using several tumor cell lines: A549 (lung cell carcinoma), HCT15 (colon cell carcinoma), MCF7 (breast adenocarcinoma), and U373 (glioma cell). Cytotoxic effects of the pharmaceutical wastewater were examined before and after the treatment with *P. putida*, in which a dose-response effect was observed for cell viability. Following cytotoxicity decreasing mainly for U373 cells, the authors have tested the genotoxic effects by comet assay using this cell line. According to them, the genotoxicity found to non-treated samples is result from synergistic actions given by antibiotic compounds present in the wastewater. However, after treating the effluent with bacteria the genotoxic effect diminished significantly, proving the effectiveness of aerobic biodegradation and the suitability of human cell culture as a sensible model for this purpose [65].

As can be observed, mammal and human cell culture comprise a useful tool to check biodegradation processes. Nevertheless, there are not many studies using these techniques, since researchers give preference to assays with microorganisms or with higher plants. Despite the major acceptance of microbial and plant bioindicators, assays with cell culture should be more explored because it provides evidence closer to real effects in human health, which could complement other information obtained by another assay.

5 Final Considerations

Bioremediation is a process based on living organisms (e.g., plant, bacteria, fungi, and algae) or enzymes, whose action provides total removal or decreases the concentrations of toxic compounds present in the environment. This method is used exclusively to assist in the control of environmental pollution. This process of treatment applied to impacted environments has been gained attention by the fact to not cause or cause less secondary pollution. Also, it can be established to recover and assess environments (aquatic—surface and groundwater—and edaphic) or industrial residues or wastewater. Although this process is considered the best cost-benefit among the remediation processed and could be applied to a broad variety of toxic organic contaminants, there are some physicochemical factors that could limit its implementation, such as pH, salinity, redox potential, temperature, and humidity. Despite these factors, there are others related to the contaminant chemical structure, with the presence of other compounds and with the bioavailability of the substance. Thus, each bioremediation process is unique and, in

almost all cases, it requires an adjustment and a specific optimization before the application. Consequently, whenever a bioremediation is applied, it is necessary to perform a prior and integrated analysis of the physical, chemical, and biological parameters involved in the process, such as the contaminant biodegradation potential, the suitable substrate for the development of degrading organisms, the presence of microorganisms with effective enzymatic activity to degrade the target, and proper temperature to activate and maintain the degradation. For these reasons, every biodegradation process should pass through a careful monitoring in all stages to assure the yield of the implemented method.

Several bioremediation processes used today to degrade chemical pollutants are harmful to biological environments, as discussed in this chapter.

Generally, the efficiency assessment of a bioremediation process is performed by comparative chemical analyses between the initial and the final stage. However, these analyses contemplate only the factors related to the contaminant chemical features. In order to know the risks of a contaminant to a living organism, it is necessary to carry out biological assays that could estimate the real effects of the xenobiotic on the biological environment. Living organisms present diverse sensibility to toxic agents. Biological assays used to measure toxicity caused by an agent must be developed with highly sensitive organisms, known as bioindicators. Due to that, the choice of which bioindicator should be used is essential to guarantee the quality of the assessment. A good strategy to perform a correct estimation of bioremediation yield is based on a battery of bioassays that uses organisms from different taxonomic groups and distinct trophic levels, responding to miscellaneous routes of action and exposure periods.

Many are the bioassays used to investigate the effectiveness of bioremediation. Among them, microorganisms, plants, and mammals, including cultured human cells, are the most used organisms due to their great sensibility to chemical toxic compounds, as shown in Table 1.

In this chapter was discussed the using of prokaryotes, such as *Escherichia coli*, *Salmonella typhimurium,* and *Vibrio fischeri*. These species are the most employed in bioassays to assess bioremediation through tests such as Microscreen, SOS-Chromotest, Amest test, Mutatox® and Microtox®, which are designed to verify toxic and mutagenic potential.

Plants are recognized bioindicators of toxic effects by chemical compounds, for this reason these are widely used to evaluate bioremediation. The species mentioned in this chapter (*Allium cepa*, *Arabidopsis thaliana*, *Capillaris crepis*, *Glycine max*, *Hordeum vulgare*, *Lactuca sativa*, *Nicotiana tabacum*, *Tradescantia* sp., *Vicia faba,* and *Zea mays*) are applied in tests of toxicity (germination and growth rates), cytotoxicity (mitotic index and cellular death),

Table 1
Compilation of the bioassays used to assess the efficacy of biodegradation of environmental contaminants discussed in this chapter

Sample/contaminant	Method	Bioassay	Endpoint	References
Weathered drill wastes contaminated with petroleum hydrocarbon	In situ remediation and biopreparation	Ames and Microtox®	Mutagenicity and toxicity	Steliga et al. [34]
Sewage Sludge	Monitored natural attenuation	Kado with TA 98 and TA 100 strains Micronuclei test with HepG2 cells	Mutagenicity	Mazzeo et al. [35]
Acid Red 114 (Azo dye)	Biodegradation using microorganism enrichment culture	Ames test TA 98 and TA 100 strains	Mutagenicity	Corroqué [36]
Textile wastewater	Ligninolytic enzymes (*Ganoderma lucidum*)	Ames test TA 98 and TA 100 strains	Mutagenicity	Bilal et al. [37]
Micropollutants (ibuprofen, clofibric acid and carbamazepine)	Biodegradation using microorganism enrichment culture	Microtox®	Toxicity	Marco-Urrea et al. [38]
Herbicides (alachlor, diuron, glyphosate and some metabolites)	Pure herbicides and its known metabolites	Microtox®	Toxicity	Bonnet et al. [26]
Municipal sludge	Vermicomposting	*Allium cepa* test	Phytotoxicity (root elongation test) and genotoxicity (mitotic and chromosomal aberrations)	Srivastava et al. [52]
Sewage sludge	Monitored Natural Attenuation	*Allium cepa* test	Cytotoxicity (mitotic index), genotoxicity (chromosomal aberrations and nuclear abnormalities) and mutagenicity (micronuclei and chromosomal breakages)	Mazzeo et al. [51]

(continued)

Table 1 (continued)

Sample/contaminant	Method	Bioassay	Endpoint	References
Sewage sludge	Composting with wood shaving	Toxicity evaluation in *Hordeum vulgare and Lepidium sativum*	Seeds germination	Mena et al. [53]
Petroleum hydrocarbons	Landfarming, before and after the addition of rice hulls	*Allium cepa* test	Mitotic and chromosome abnormalities, micronucleus and nuclear buds	Souza et al. [54]
Polycyclic aromatic hydrocarbons (PAH)	Biobed	*Growth test with Brassica rapa and Avena sativa*	Weight (biomass production)	Hund and Traunspurguer [4]
Soils contaminated with creosote	Microcosms	*Toxicity evaluation in Lactuca sativa*	Seeds germination	Phillips et al. [49]
Benzene, toluene, ethylbenzene and xylene (BTEX)	Biodegradation using microorganism enrichment culture	*Allium cepa test*	Genotoxicity (chromosomal aberrations) and mutagenicity (micronuclei)	Mazzeo et al. [50]
Industrial effluents	Algal biofilters	*Phytogenotoxicity test with Allium cepa and Vicia faba*	Cytotoxicity (mitotic inhibition) and genotoxicity (chromosomal aberrations and nuclear irregularities)	Migid et al. [55]
Pulp and paper mill effluent	Bioaugmentation	*Toxicity evaluation in Vigna radiata L.*	Seeds germination	Raj et al. [56]
Azo dye (black dye commercial product—CDCP)	Biodegradation using microorganism enrichment culture	*Allium cepa test*	Cytotoxicity (apoptotic and necrotic cells), genotoxicity (chromosome and nuclear aberrations) and mutagenicity (micronuclei and chromosome breakages)	Ventura-Camargo et al. [57]
Herbicide atrazine	Bioaugmentation and biostimulation	*Toxicity test with Avena sativa*	Plant biomass production	Chelinho et al. [58]

Sugarcane filter cake	Bioremediation with and without addition sugarcane bagasse and soil	*Allium cepa test*	Cytotoxicity (mitotic index), genotoxicity (chromosomal abnormalities) and mutagenicity (micronuclei)	Anacleto et al. [59]
Dyes (azo and triphenyl dyes)	Biodegradation of azo and triphenylmethanes dyes using Staphylococcus epidermidis isolated from industrial wastewater	*MTT assay with Hep-2 cells*	Cytotoxicity	Ayed et al. [64]
Direct Red 28—DR28 (dye)	Biodegradation using enrichment with microorganism culture (Bacillus velezensis)	*Flow cytometry, TUNEL assay and comet assay with HL-60 cells*	Cytotoxicity and genotoxicity	Bafana et al. [83]
Paper mill sewage sediment	Dichloromethane/acetone extraction followed by treatment with indigenous Pseudomonas sp. strain ISTDF1	*MTT assay and comet assay with HuH-7 cells*	Cytotoxicity and genotoxicity	Das et al. [84]
Paper mill black liquor	Hexane/acetone extraction followed by treatment with Bacillus sp.	*MTT assay and comet assay with HuH-7 cells*	Cytotoxicity and genotoxicity	Mishra et al. [85]
Electroplating effluent	Treatment with activated charcoal and treatment with indigenous bacteria Bacillus sp. strain IST105	*MTT assay and comet assay with HuH-7 cells*	Cytotoxicity and genotoxicity	Naik et al. [86]
Benzene, toluene, ethylbenzene and xylene (BTEX)	Biodegradation using microorganism enrichment culture	*Micronuclei test and comet assay with HTC cells*	Genotoxicity and mutagenicity	Mazzeo et al. [60]
Cypermethrin (insecticide)	Soil microcosms with Bacillus sp. strain ISTDS2	*MTT assay and comet assay with HuH-7 cells*	Cytotoxicity and genotoxicity	Sundaram et al. [87]
Endosulfan (insecticide)	Soil microcosms with Paenibacillus sp. strain ISTP10	*MTT assay and comet assay with HepG2 cells*	Cytotoxicity and genotoxicity	Kumari et al. [66]
Pharmaceutical wastewater	Treatment with P. putida mt-2 (DSM 3931)	*MTT assay with cell culture (A549, HCT15, MCF7 and U373 lineages)* *Comet assay with U373 cells*	Cytotoxicity and genotoxicity	Krifa et al. [65]

genotoxicity (chromosomal aberrations, nuclear abnormalities, and sister-chromatid exchange), and mutagenicity (presence of micronucleus, modification of chromosomal constitution, and distribution of the genetic material) relevant to environmental impact assessment before and after bioremediation.

Although the in vitro bioassays are less explored in bioremediation assessments, they present great sensibility to these experiments. Thus, this chapter reviewed some examples of bioassays based on cells culture that showed very satisfactory results. From these assays it is possible to evaluate several endpoints, as cytotoxicity (e.g., MTT test and neutral red retention test), genotoxicity (comet assay), and mutagenicity (CBMN and CBMN-Cyt test). Therefore, this chapter intends to provide information about the effectiveness of these assays and recommend its using to certify bioremediation, as they are extremely suitable for these analyses.

As bioremediation in itself cannot guarantee the full transformation of a xenobiotic to minerals or less harmful compounds, it is necessary to execute biological analyses in all the steps of the process to assure the detoxification.

Thus, this chapter tried to gather data about: (1) some types of bioremediation, to provide more procedural options of this type of degradation; (2) test-organisms or bioindicators effective to assess bioremediation processes; and (3) bioassays that could evaluate the most variable endpoints, which allows covering all forms of toxicity that an agent could present.

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Chapter 12

New Approach of Dye Removal in Textile Effluent: A Cost-Effective Management for Cleanup of Toxic Dyes in Textile Effluent by Water Hyacinth

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Abstract

Effluent from textile, paper, plastic, leather, and cosmetic industries are the major concern in the aspect of environmental toxicities. Many complex and aromatic dyes due to their incomplete degradation and accumulation exhibit toxic effects in aquatic life and human health. Currently, there are many dye degradation treatment plants that are being operated to reduce their toxicity but the major limitation is their cost. Therefore, in search of the most efficient and reliable method of removing pollutants, a non-conventional cost-effective biosorbent (water hyacinth) was used in this experiment. In this chapter, it is discussed about the removal of dyes from textile effluent by degrading the dyes using the cheapest method of utilizing a widely available water hyacinth plant. This plant was pretreated with phosphoric acid to increase the porosity. Three dyes (AR97, AB20, and AY19) were tested for their removal by water hyacinth. The batch adsorption experiment was carried out to determine equilibrium behavior. Effects of operating parameters like initial dye concentration, sorbent dosage, contact time, and temperature on the sorption efficiency were also studied. Adsorption isotherm models were also used to simulate the equilibrium data at different experimental parameters. Finally, it was concluded that water hyacinth exhibits maximum decolorization efficiency (nearly 99%), thus reducing the toxicity of textile dyes.

Key words Toxicity reduction, Textile effluent, Dyes, Biosorbent, Water hyacinth, Adsorption isotherm models, Decolorization

1 Introduction

1.1 Background Information

Water is the major indispensable element essential for all the lives on earth. Due to growing human population and their activities, the rising agony all over the world is chemical pollution of water. The eventual outcome of toxicity of chemicals in water is two significant factors—"bioaccumulation" and "biomagnification."

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– Bioaccumulation—accumulation of a greater amount of toxic chemical in an organism that exceeds that of the surrounding environment.
– Biomagnification—increased concentration of toxic chemicals in food chain including human beings. Toxic chemicals from surroundings reach the water surface through various ways such as effluent from several industries, such as textile, paper, plastics, and dyestuffs, since they generate a large volume of polluted wastewater [1]. Normally, these chemicals will be degraded by several aquatic plants in the ponds, rivers, and seawater. But when the toxicity exceeds a certain limit it becomes nuisance to demolish.

1.2 Dyes

Dyes are widely used in textile, paper, printing industries, and dye houses. All over the world, about 60,000 tons of dyes are released in wastewater every year [2]. There are about 1 lakh commercially available synthetic dyes [3, 4]. Among all the water polluting effluent, textile effluent is unbearable and poisonous one [5]. Wastewater from textile industry is crammed with large amount of various coloring dyes. The dark color of the dyes in water system blocks the sunlight and this subsequently affects the aquatic plants and animals, and thus greatly affects the ecosystem [6]. Dyes are found to be non-biodegradable by aerobic treatment systems. Dyestuffs consist of 50% dye by weight, sugars, surfactant, and some heavy metals such as Pb, Hg, Cr, Cu, and Zn. These heavy metals enter the ecosystem through plants and animals by assimilation which has a great impact on the central dogma of life. The two foremost components of dye molecules are chromophores and auxochromes. Chromophores are responsible for producing the color and the auxochromes supplement the chromophores. The key feature of the dyes to exhibit non-biodegradability is that they are aromatic compounds with a heterocyclic molecular structure. The textile dyes are mostly resistant to fading by chemicals and light due to this nature of dyes. Textile dyes are also designed to be resistant to fading by chemicals, high temperature, enzyme degradation, and light.

Dyes are classified as the following types:

– Anionic: direct, acid, and reactive dyes;
– Cationic: basic dyes;
– Nonionic: disperse dyes [7].

Basic dyes are usually toxic because of their cationic nature. Direct dyes are nontoxic. Generally, from a dye class standpoint, basic dyes appear to be the most toxic because of their cationic nature. The remaining dyes are not directly toxic and may be converted into carcinogenic compounds by anaerobic digestion in

landfill sites. Thus, all the dye compounds are potentially poisonous. The azo groups and anthraquinone types present in the chromophores of anionic and nonionic dyes are reduced to toxic amines in the effluent [8]. Anthraquinone is more challenging to degrade since they have fused aromatic structures and thus remain colored for a longer time. Reactive dyes provide greater stability to fabrics as they have different types of reactive groups, e.g., vinyl sulfone, trichloropyrimidine, chlorotriazine, difluorochloropyrimidine. These reactive groups form covalent bonds with oxygen, nitrogen, and/or sulfur atoms present in cellulose fibers, protein fibers, and polyamides [9].

1.3 Effluents

Textile industry effluent is the foremost irritant released into the environment. It consists of dyestuffs (aromatics), heavy metals, starches, gums, resins, waxes, and other organic, inorganic, and aromatic compounds. Dark colored dyes block the penetration of sunlight into the water system such as pond, lakes, etc. It significantly affects the photosynthetic activity in aquatic life, which in correlation increases the oxygen demand of many plants and animals. Liquor released from various dyeing processes causes various troubles. Liquor obtained from sulfur dyeing consists of sodium sulfide which poses staining. Chrome dyeing results in liquor that contains heavy metals that interact with biochemical process and this may be fatal. Dyeing of fabrics with acidic and alkaline dyes results in liquor with large amount of salts and surface active agents. Dye effluent causes a major bang on human health due to toxic, carcinogenic, mutagenic, and/or teratogenic effects [10].

1.4 Challenges in Treatment

Effluent treatment is one of the most challenging procedures for the experts. Untreated wastewater creates numerous problems to the environment.

Purpose of effluent treatment processes:

1. Reduction of BOD and COD of wastewater.
2. Removal of organics and dyes those are toxic or carcinogenic.
3. Removal/reduction of toxic heavy metals.
4. Reduction of nutrients (N, P) to prevent the contamination by microbes.
5. Destruction of pathogenic microbes.

1.4.1 Biochemical Oxygen Demand (BOD)

It is defined as the amount of dissolved oxygen (DO) consumed by microbes for the biochemical oxidation of organic (carbonaceous BOD) and inorganic matter (autotrophic or nitrogenous BOD). The maximum limit of BOD in water stream is 8 mg/L. If it exceeds this limit, it should be diluted. The BOD test was used to predict the effect of wastewater on receiving streams and determine their capacity to assimilate organic matter [11]. It is the first test

done to determine the quality of wastewater and evaluate the impact of the plant effluents on receiving waters.

1.4.2 Chemical Oxygen Demand

Chemical oxygen demand (COD) is defined as the amount of oxygen needed to oxidize the organic carbon absolutely to CO_2, H_2O, and ammonia [12]. COD is measured by means of oxidation with potassium dichromate ($K_2Cr_2O_7$) in the presence of sulfuric acid and silver and its unit is mg/L. Thus, COD is a measure of the oxygen equivalent of the organic matter as well as microorganisms in the wastewater [13].

1.4.3 Common Treatments and Restrictions

Effluent treatments commonly used to remove the dyes are biological oxidation and chemical precipitation. These include chemical flocculation, oxidation, ozonation, irradiation, coagulation, adsorption, precipitation, and ion exchange. Although they are effective, there are some limitations. These include enormous usage of chemicals for treatment, accumulation of these chemicals in treated water, disposal problems of these accumulated chemicals, expensive infrastructure, operational cost, and lack of complete color reduction [14].

1.4.4 Alternative Treatments

Alternative treatment that includes adsorption by activated carbon has been revealed to be the most hopeful option for the removal of non-biodegradable dyes from aqueous streams due to its effectiveness. Activated carbon has high carbon content and greatly adsorbs the chemical due to porous nature. In spite of many advantages, there are disadvantages too. They are quite expensive and both chemical and thermal regeneration of spent carbon is expensive, impossible on a large scale [15]. All these constraints create a pressure to search for cheap and effective adsorbent. Phytoremediation technologies based on the principle of utilizing plants and their biomass as biosorbent is very much cost effective, ecofriendly, and socially acceptable methods compared to other physical and chemical methods.

1.5 Phytoremediation

There are three major mechanism involved in Phytoremediation technology.

1. Rhizofiltration.
2. Phytovolatilisation.
3. Phytostimulation.

In rhizofiltration, chemical pollutants are absorbed, filtered by the roots of aquatic plant and get concentrated. These concentrates are chemically modified as their carbonates and phosphates. Whereas in phytovolatilization, the chemical pollutants were absorbed by the aquatic plants, later they are transpired by their aerial organs.

Phytostimulation involves the degradation of chemical pollutants by the microorganisms present in the roots of aquatic weeds. The area of the roots where an increased activity of chemical degradation by microbes takes place is called rhizosphere. Stimulation of these areas by introducing microbiologically active rhizospheres in the aquatic system resulted in a massive degradation of pollutants and this process is referred to as phytostimulation. These microbes exhibit a symbiotic relationship. The roots of the plants produce exudates such as amino acids, sugar, essential vitamins, and enzymes. Microbes use these compounds and chemical pollutants as their substrate uses enzymes as their catalyst for biochemical activities. Thus, symbiosis resulted in degradation of pollutants, availability of nitrogen to plants, and production of substrate for microorganisms, e.g., Water hyacinth [16].

1.6 Mechanism Behind Biodegradation

1.6.1 Phytodegradation

Microorganisms present in the roots and shoots of plants produce enzymes such as dehydrogenases, oxygenases, and reductases both intra and extra cellularly. In case of intracellular enzymes, species absorb the chemicals first and then degrade intra cellularly. In case of extracellular, species directly act on the chemicals and degrade. The biodegraded compounds are later converted into insoluble and inert materials such as amino acids, sugars, etc., that are stored in the lignin or released as exudates.

1.6.2 Phytotransformation

In case of phytotransformation, plants absorb the pollutants. Microorganisms produce enzymes and convert the chemicals into other forms (carbonates, phosphates) by means of biotransformation. These chemicals can be later used as nutrients for plants and microbes. Some of the dry biomass of aquatic plants that are useful in the treatment of textile effluents are listed in Table 1.

Table 1
Use of dry biomass of aquatic plants for treatment of textile effluents

S. No.	Plant	Scientific name	Chemicals degraded
1.	Indian Mustard	*Brassica junceae*	Toxic heavy metals (Pb, Cd, Cr, Cu and Ni) [36] Lead (Pb) from aqueous solutions in the range of 4–500 mg/L [37]
2.	Sunflower	*Helianthus anus*	Radionucleides cesium (Cs), strontium (Sr) and uranium (U) [38]
3.	Vetiver Grass	*Vetiveriazizaniodes*	Heavy metals AI, Mn, Mg, As, Cd, Cr, Ni, Cu, Pb, Hg, Se, Zn [39].
4.	Duckweeds	*Lemnagibba*	Dissolved gases, phosphorus, nitrate and potassium [40].
5.	Water hyacinth	*Eichorniacrassipes*	Lead (Pb), cadmium (Ca), nickel (Ni), chromium (Cr), zinc (Zn), copper (Cu), iron (Fe) and pesticides and several toxic chemicals from the sewage. [41]

1.7 Water Hyacinth

Among the widely used plants for phytoremediation, researchers are now focusing on water hyacinth for their rapid production rate and efficient chemical adsorption properties [17]. Water hyacinth (*Eichornia crassipes*) are generally considered deleterious hydrophytes filling the ponds, lakes, and wetlands, as they block the sunlight and increase BOD of the water system. But scientifically water hyacinth performs the most of valuable activity to the environment by degrading the pollutants in the water bodies. These aquatic weeds are green colored nature characterized by large, flat leaves and a long spongy petiole. They are partially submerged in water seems as if they are floating in water. The fibrous root system of the plant gives their tight grip to anchor with the soil. Microorganisms present in the water hyacinth mainly in their roots exhibit a symbiotic relationship by utilizing the toxic chemicals and minerals as their feed, thus degrading them from the effluent. In turn by digesting the toxics they release sugars and amino acids that are used by the plants to grow. Further, the plant supplies oxygen and nutrients to the microbes for their biochemical action and this regulation helps to standardize carbon dioxide level by photosynthesis. So far much research had been done to study dye degrading property of water hyacinth. The present research focused on modifying the surface of water hyacinth in order to effectively utilize it as a biosorbent.

2 Materials

Cotton Red B2G dye [AR97], Cotton Blue B2G dye [AB20], Cotton Yellow 2RFL dye [AY19] were purchased from Gaiety fashions, Tirupur, Tamil Nadu, India. These dyes were used in this study without further chemical changes to assess the efficiency of phosphoric acid treated water hyacinth stem as an effective biosorbent. Physico-chemical characteristics of these three dyes were explored in Table 2. Analytical grade Phosphoric acid was purchased from SD chemicals, Tamil Nadu.

3 Methodology

3.1 Determination of λmax and Calibration Curve for Three Different Dyes

λmax of the three different dyes [AR97, AB20, and AY19] were determined by scanning the dye solutions using UV-Visible spectrophotometer. A sample of 10 μg/ml concentration was taken for each dye. λmax values of the respective dyes were given in Table 2. Optical density values at different concentrations of three dyes at their corresponding λmax were estimated using UV-Visible spectrophotometer. Calibration curves were plotted for the dyes to determine the absorbance for the corresponding concentration.

Table 2
Physico-Chemical Characteristics of the dye

S. No	Dyes	λmax (nm)	Structure	Mol. wt	Formula
1.	Cotton Red dye B2G (AR97)	595		698.63	$C_{32}H_{20}N_4Na_2O_8S_2$
2.	Cotton Blue dye B2G (AB20)	535		416.38	$C_{20}H_{13}N_2NaO_5S$
3.	Cotton Yellow dye 2RFL (AY19)	415		601.35	$C_{20}H_{12}C1_2N_4Na_2O_7S_2$

3.2 Preparation of Biosorbent

Eichornia crassipes (Water hyacinth—WH) used in this study was collected from river banks near Thiruverumbur, Trichy, Tamilnadu, India. The collected plant was authenticated by Dr. Soosairaj, Assistant Professor, Dept. of Botany, St. Joseph's College, Trichy-2. Voucher specimen is preserved at Dept. of Pharmaceutical Technology for further access. The plant parts [stem, leaves, and roots] were separated and thoroughly washed with tap water to remove earthy materials and then washed with distilled water. Plant parts were cut into small pieces and dried under sunlight for about 4 days. Dried parts were grinded into powder and sieved through 40 mesh size to obtain even particles [18]. Powdered WH was evaluated for dye removal efficiency.

3.3 Preliminary Batch Experimental Procedure to Determine Contact Time

Experiments were initiated from small scale using 100 mL Erlenmeyer flask. 25 mL of three different dye solutions [AY97, AY19, and AB20] each of concentration 10 mg/L were taken separately in three flasks. Contact time required to attain equilibrium state of dye removal is the major intrinsic factor to be determined before all other experiments. To evaluate the effect of contact time, the reaction was performed using sorbent dosage (untreated water hyacinth stem part) of 0.4 g/L. Dye solution and water hyacinth were mixed by placing the flasks in a rotary shaker at 150 rpm and 25 °C. For each dye, mixtures were withdrawn at regular time interval of 10 min and the adsorbate was separated by centrifuge. The concentrations of the residual dye solutions in the supernatant

were measured at their corresponding wavelength (λmax) [19]. At a certain period for all the dyes, absorbance reaches equilibrium, at which the experiment was stopped and readings were evaluated. Equilibrium is the condition under which there is an equal distribution of dye between the solid and fluid phases and no further adsorption occurs. The experiment was conducted in triplicate and mean values of data were reported. Standard deviations were expressed as error bars in the graphical figures.

The amount of dye adsorbed by WH (mg of dye/mg of WH) at any time t is Q_t. It was calculated as

$$Q_t = \frac{V[C_\mathrm{i} - C_t]}{W}$$

At equilibrium, $Q_t = Q_\mathrm{e}$ and $C_t = C_\mathrm{e}$,

$$Q_\mathrm{e} = \frac{V[C_\mathrm{i} - C_\mathrm{e}]}{W}$$

C_i = initial concentration of dye [10 mg/L]
C_t = concentration of dye at any time t [mg/L]
C_e = equilibrium concentration of dye [mg/L]
V = volume of solution [L]
W = mass of adsorbent [g]
Dye removal efficiency of WH was calculated as

$$[R] = \left\{\frac{[C_\mathrm{i} - C_t]}{C_\mathrm{i}}\right\} \times 100$$

3.4 Adsorption Kinetic Study

The adsorption kinetic study was performed to identify whether the dye adsorption by *Eichhornia* follows first order or second order. The study was carried out for a single dye AR97 and the mean values of Q_e, Q_t, and t were taken from the contact time experiment.

The pseudo-first-order equation is known as Lagergren equation [20],

$$\frac{\mathrm{d}Q_t}{\mathrm{d}t} = K_\mathrm{f}(Q_\mathrm{e} - Q_t)$$

K_f (min^{-1}) is the pseudo first-order rate constant, and t is the contact time (min).

On integrating the above equation from time zero to t, it gives

$$\log(Q_\mathrm{e} - Q_t) = \log Q_\mathrm{e} - \frac{K_\mathrm{f}}{2.303}t$$

The plot of log ($Q_\mathrm{e} - Q_t$) vs t gives the slope $-\frac{K_\mathrm{f}}{2.303}$ and intercept log Q_e.

The pseudo-second-order model is characterized as the Ho and Mckay equation [21].

$$\frac{dQ_t}{dt} = K_s(Q_e - Q_t)^2$$

where K_s is the pseudo second-order rate constant (mg mg^{-1} min^{-1}).

Integrating

$$\frac{t}{Q_t} = \frac{1}{K_s Q_{e^2}} + \frac{1}{Q_e} t$$

The equilibrium adsorption capacity, Q_e, is obtained from the slope and K_s is obtained from the intercept.

3.5 Effect of Plant Part on Dye Removal Efficiency

Twenty-five milliliters of three different dye solutions [AY97, AY19, and AB20] each of concentration 10 mg/L were taken separately in three flasks each. 0.4 g/L concentration of plant parts (stem, root, and leaves) of untreated water hyacinth was added to each dye solution separately. As per the result of the contact time procedure, the experiment was carried out for such time and readings were taken as above. Mean values were calculated. The efficient plant part that removes greater amount of dye was identified and used further.

3.6 Chemical Treatment of Water Hyacinth

From the batch experimental procedure to determine efficient plant part, the part that exhibits maximum dye removal efficiency was found. This part was chemically modified to increase the porosity by treatment with phosphoric acid (H_3PO_4) to estimate the improved efficiency of dye removal. About 20 g of raw water hyacinth stem was soaked in 50 mL phosphoric acid at the ratio of 2:5 and kept in a mechanical stirrer at 100 °C with constant stirring for 2 h. After this treatment, the sample was thoroughly washed with distilled water. Later, they were dried in a hot air oven at 100 °C and stored in a desiccator for further use. The resulting sample was referred to as H_3PO_4 treated water hyacinth (PAWH) [22]. Dye removal efficiency of untreated WH and PAWH was determined by following the above similar procedures of using WH adsorbent dosage of 0.4 g/L for initial dye concentration of 10 mg/L (volume 25 mL), and contact time 60 min.

3.7 Effect of Initial Dye Concentration

Dye removal efficiency and contact time to reach equilibrium concentration depend on major factor "Initial dye concentration." The effect of initial dye concentration on the adsorption capacity of PAWH was investigated as follows. 25 mL of three different dye solutions as described above with a concentration ranging from 5 to 30 mg/L were taken separately in conical flasks. 0.4 g/L of efficient plant part treated with phosphoric acid was added to the entire

flasks and the same procedures as above were followed. The amount of dye adsorbed (Q_e) by the PAWH after reaching equilibrium state (i.e., after 60 min) for each initial dye concentration was calculated and their corresponding equilibrium concentrations C_e were also determined. These data were used to examine the adsorption isotherm models and isotherm constants.

3.8 Effect of Adsorbent Dosage on Dye Removal

Dye removal efficiency, amount of dye adsorbed, and time to reach equilibrium concentration depend on another major aspect "adsorbent dosage." To investigate the effect of adsorbent mass, different masses of PAWH (0.1–1 g/L) were introduced to thirty conical flasks (ten flasks for each dye) containing a specific volume 25 ml of fixed initial concentration (10 mg/L) of three dyes. Concentrations of residual dye were measured after reaching equilibrium at 60 min [19].

3.9 Biosorption Isotherms

Results of adsorption equilibrium are characterized by Isotherm models and their equations and further used for design purposes. The most resourceful adsorbent and their performance can be predicted by adsorption isotherms. The equilibrium relationship between the dye solutions in PAWH and in liquid phase can be well represented by isotherm models. The commonly used isotherm models are Langmuir, Freundlich, and Tempkin isotherms. However, no single model is universally applicable; all the models engage assumptions that may or may not be valid in particular cases [23].

One of the simplest adsorption isotherm models that precisely portray certain practical systems is the Langmuir isotherm. This model is based on the hypothesis that all the dye particles have equal binding site and equal energy on the adsorbent surface and the dye molecules exist as monolayer on the outer surface of PAWH where all the adsorption sites are identical.

The Langmuir equation is as follows:

$$Q_e = \frac{Q_m K_L C_e}{1 + C_e K_L}$$

where Q_e is the equilibrium dye concentration on the adsorbent (mg mg^{-1});

C_e is the equilibrium dye concentration in solution (mg L^{-1});

Q_{max} is the monolayer capacity of the adsorbent (mg mg^{-1}) or maximum adsorption capacity;

K_L is the Langmuir constant.

By modifying the Langmuir isotherm equation in various derivative forms, it can be developed into three more different isotherm equations and they are publicized in Table 3.

The other widespread used isotherm model is Freundlich isotherm. The Freundlich isotherm explains that heterogeneous

Table 3
Equation and isotherm plot for Isotherm models

Isotherm models	Linear form	Plot
Langmuir I	$\frac{C_e}{Q_e} = \left(\frac{1}{Q_m}\right)C_e + \left(\frac{1}{K_L Q_m}\right)$	$\frac{C_e}{Q_e}$ vs C_e
Langmuir II (Lineweaver Burk)	$\frac{1}{Q_e} = \left(\frac{1}{Q_m}\right) + \left(\frac{1}{K_L Q_m}\right)\frac{1}{C_e}$	$\frac{1}{Q_e}$ vs $\frac{1}{C_e}$
Langmuir III (EadieHofstee)	$Q_e = Q_m - \left(\frac{1}{K_L}\right)\frac{Q_e}{C_e}$	Q^e vs $\frac{Q_e}{C_e}$
Langmuir IV (Scatchard)	$\frac{Q_e}{C_e} = K_L Q_m - K_L Q_e$	$\frac{Q_e}{C_e}$ vs Q_e
Freundlich	$\ln Q^e = \ln K^F + \frac{1}{n} \ln C^e$	$\ln Q_e$ vs $\ln C_e$
Temkin	$Q_e = B \ln K_T + B \ln C_e$	Q_e vs $\ln C_e$

systems exist, i.e., dye molecules exhibit non-energetically equivalent binding sites. The equation is expressed as

$$\log Q_e = \log K_F + (1/n)\log C_e$$

where Q_e is the equilibrium dye concentration on the adsorbent (mg mg^{-1});

C_e is the equilibrium dye concentration in solution (mg L^{-1});

K_F is the Freundlich constant (represents performance);

$1/n$ is the heterogeneity factor (represents effectiveness).

When $1/n$ value is equal to unity, the adsorption is linear and apt;

when it is below unity, the adsorption process is unfavorable; and

when it is above unity, adsorption is favorable.

Temkin and Pyzhev developed an isotherm where the binding energy and binding site of all the dye molecules are assumed to have uniform allocation and the heat of adsorption decreases linearly with surface coverage of adsorbent due to their interactions. The linear form of the Tempkin isotherm equation is given as

$$Q_e = B \ln A + B \ln C_e$$

Where $B = RT/b$, T is the absolute temperature in K,

R the universal Gas constant (8.314 JK^{-1} mol^{-1}),

A the equilibrium binding constant,

the constant B is related to the heat of adsorption.

Values of B and A were calculated from the plot of Q_e against $\ln C_e$.

4 Results and Discussion

4.1 Preliminary Batch Experimental Procedure to Determine Contact Time

The batch experimental procedure to find reaction time to reach equilibrium resulted in that adsorption of all the three dyes onto water hyacinth stem part reached equilibrium in about 60 min for the initial dye concentration of 10 mg/L (Fig. 1). Till 40 min, the adsorbed amount onto WH stem increased steeply. After that, adsorption slowed and reached equilibrium at 60 min. Results were exposed in Table 4. The equilibrium residual dye concentrations for the three dyes AR97, AY19, and AB20 were 7.529 mg/L, 8.309 mg/L, and 7.835 mg/L, respectively. From the results it was predicted that the strength of second dye AY19 was greater compared to other two dyes and hence adsorbed slowly. The amount of dye adsorbed per milligram of WH stem for three dyes after 60 min was found to be 6.17 mg, 4.22 mg, and 5.42 mg respectively. The value of Q_t increased steeply till 40 min after this time it increased slowly. The mechanism behind this variation was as follows:

1. The concentration gradient of dye molecules between solid phase (WH) and liquid phase (distilled water) was higher initially and hence mass transfer was also greater. Thus, the Qt value was greater.
2. As the time passes, at particular point, the adsorbed dye molecules start developing a boundary layer over the adsorbent. The free dye molecules have to cross this rate-limiting step (boundary layer), and then diffuse through the surface of the

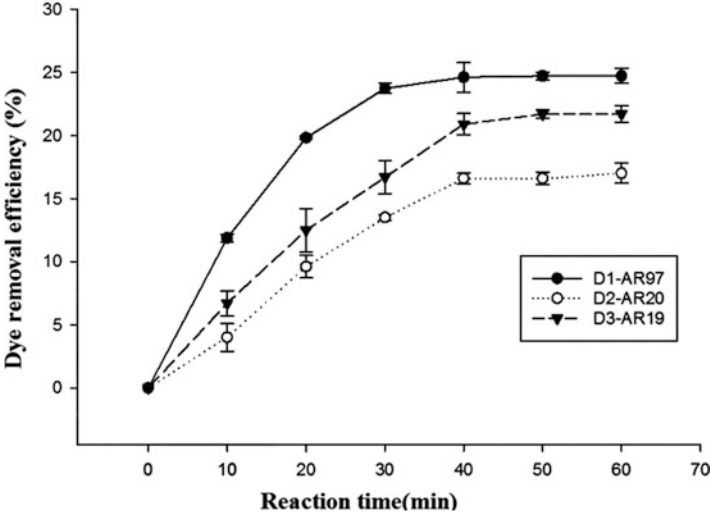

Fig. 1 Effect of contact time on dye removal efficiency for Water hyacinth on three dye solutions (WH dosage = 0.4 g/L, initial dye concentration = 10 mg/L, volume of dye solution = 25 mL, plant part used = stem)

Table 4
Effect of contact time on dye removal efficiency for Water hyacinth on three dye solutions

	C_t (mg/L)			Q_t (mg of dye sorbed/mg of WH) $\times 10^{-3}$			R (% of dye removed)		
Time (min)	D_1	D_2	D_3	D_1	D_2	D_3	D_1	D_2	D_3
0	10	10	10	0	0	0	0	0	0
10	8.813 ± 0.03	9.600 ± 0.11	9.331 ± 0.10	2.96 ± 0.07	1.00 ± 0.28	1.67 ± 0.25	11.87 ± 0.3	4.0 ± 1.1	6.7 ± 1.01
20	8.019 ± 0.01	9.040 ± 0.09	8.752 ± 0.17	4.95 ± 0.03	2.40 ± 0.23	3.12 ± 0.43	19.81 ± 0.1	9.6 ± 0.9	12.5 ± 1.72
30	7.627 ± 0.04	8.654 ± 0.02	8.333 ± 0.13	5.93 ± 0.10	3.37 ± 0.06	4.17 ± 0.23	23.73 ± 0.4	13.5 ± 0.24	16.7 ± 1.33
40	7.539 ± 0.12	8.340 ± 0.04	7.914 ± 0.08	6.15 ± 0.30	4.15 ± 0.10	5.22 ± 0.22	24.61 ± 1.2	16.6 ± 0.4	20.9 ± 0.84
50	7.529 ± 0.03	8.340 ± 0.05	7.835 ± 0.03	6.17 ± 0.07	4.15 ± 0.13	5.42 ± 0.09	24.71 ± 0.3	16.6 ± 0.5	21.7 ± 0.35
60	7.529 ± 0.06	8.309 ± 0.01	7.835 ± 0.06	6.17 ± 0.15	4.22 ± 0.02	5.42 ± 0.16	24.71 ± 0.6	17 ± 0.79	21.7 ± 0.65

D_1, D_2, D_3 = Dyes AR97, AY19, AB20 respectively.
C_t = Concentration of the three dyes at that time t. (mg/L)
WH dosage = 10 mg/25 ml, initial dye concentration = 10 mg/L, volume of dye solution = 25 mL, plant part used = stem

adsorbent to reach the central core of the adsorbent. This will take some time and hence, the adsorption of dyes decreased gradually.

3. Later at some point, the amount of dye in solid phase and liquid phase reaches dynamic equilibrium and no more dye molecules can be adsorbed by the boundary layer. In this event, this dynamic equilibrium was achieved after 60 min for all the three dyes.

Graphical representation was shown for reaction time against dye removal percentage. The maximum percentages of dye removal by WH stem for three dyes were 24.71%, 17.00%, and 21.70 % respectively. Dye removal efficiency for AY19 was lesser since its strength was greater. Finally, it was concluded that 60 min of reaction time can be fixed to determine other optimum conditions, since the adsorption reaches equilibrium by 60 min. Similarly, the adsorption of Acid Red 114 dye on water hyacinth root increased sharply during first 45 min and reached equilibrium [24]. In the same way, M.I. El-Khaiary observed that adsorption of methylene blue dye on nitric acid treated water hyacinth reached equilibrium after 100 min at 27 °C [25]. The major reason for the initial rapid phase in all the cases may be due to abundant binding sites of the adsorbent available at the initial stage which was also shown by an experiment of methylene blue adsorption by WH that reached equilibrium in 30 min, 25 min, 15 min at 303 K, 308 K, and 313 K, respectively [26].

4.2 Adsorption Kinetic Study

The adsorption kinetic study carried out for AR97 dye using untreated WH stem part gave the nonlinear regression results and they were tabulated in Table 5. The plot for the first order was shown in Fig. 2a and for the second order was shown in Fig. 2b. Kinetic constants K_f and K_s were obtained from the plots. This study revealed that the correlation coefficient value determined for Pseudo second order ($R^2 = 0.978$) was greater than the value for Pseudo first order ($R^2 = 0.935$). Thus, it was predicted that the pseudo second-order model fitted well with the sorption mechanism. Therefore, the adsorption of AR97 dye by water hyacinth stem followed the pseudo second-order kinetic model than the first-order kinetic model. The higher R^2 value for the second-order model indicated that the chemisorptions played a major role in the dye adsorption mechanism [25]. The resulting equations for the first and second orders from the rate constant values were found as

Lagergren's Equation—first-order model

$$\frac{\mathrm{d}Q_t}{\mathrm{d}t} = 0.140((\mathrm{mg/mg})/\mathrm{min})\left(6.17 \times 10^{-3}\,\mathrm{mg/mg} - Q_t\right)$$

Table 5
Kinetic parameters for Pseudo First-order and Second-order model

Kinetic model	X axis Time (min)	Y axis	Slope	Intercept	R^2	Rate constant
Pseudo Ist order	T	$\log (Q_e - Q_t)$	$-\frac{K_f}{2.303}$ ((mg/mg)/min)	$\log Q_e$		K_f ((mg/mg)/min)
	0	−2.209	−0.061	−1.965	0.935	0.140
	10	−2.493				
	20	−2.913				
	30	−3.619				
	40	−4.698				
	50	0				
	60	0				
Pseudo IInd order	T	t/Q_t (min/(mg/mg))	$\frac{1}{Q_e}$ (mg/mg)	$\frac{1}{K_s Q_{e^2}}$ (min/(mg/mg))		K_s (mg mg^{-1} min^{-1})
	0	–	129.6	1598	0.978	10.51
	10	3378				
	20	4040				
	30	5059				
	40	6504				
	50	8103				
	60	9724				

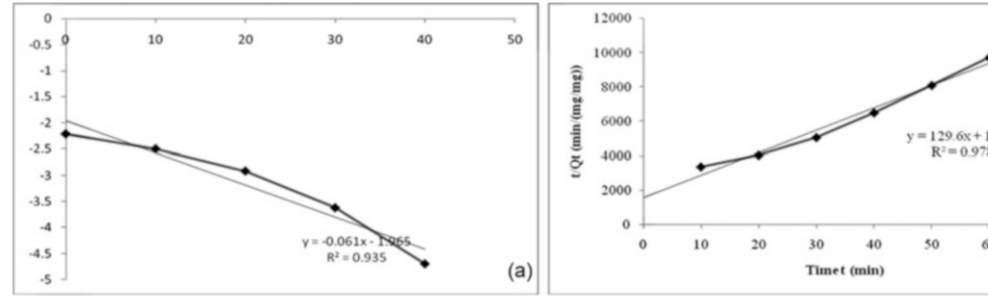

Fig. 2 (**a**) Pseudo-first-order kinetic plots for the removal ofAR97 on untreated Water hyacinth stem part (WH dosage = 0.4 g/L, initial dye concentration = 10 mg/L, volume of dye solution = 25 mL). (**b**) Pseudo-second-order kinetic plots for the removal ofAR97 on untreated Water hyacinth stem part (WH dosage = 0.4 g/L, initial dye concentration = 10 mg/L, volume of dye solution = 25 mL)

H.O equation—second-order model

$$\frac{dQ_t}{dt} = 10.51\ (\text{mg}/(\text{mg}\,\text{min}))\big(6.17 \times 10^{-3}\text{mg/mg} - Q_t\big)^2\big].$$

Likewise, for basic blue dye on *Eichhornia*, the sorption can be approximated more appropriately by the pseudo second-order kinetic model than the first-order kinetic model [26].

4.3 Effect of Plant Part on Dye Removal Efficiency

Dye decolorization using various parts of *Eichornia* has shown that dye removal efficiency was appreciably greater for stem of water hyacinth than root and leaves (Fig. 3). The results were elaborated in Table 6. Percentage of dye removed by stem part for all the three dyes ranges from 15 to 25%, for root it ranges from 5 to 16% and for leaves it ranges from 0 to 13%. Thus, the stem of WH was proved efficient and it was used further.

The reason behind the improved adsorption of dyes might be due to the nature of cells present in stem. The porosity of stem cells may be greater compared to root and leaves. Further, the higher fiber content in root and leaves resulted in improved cross linkage

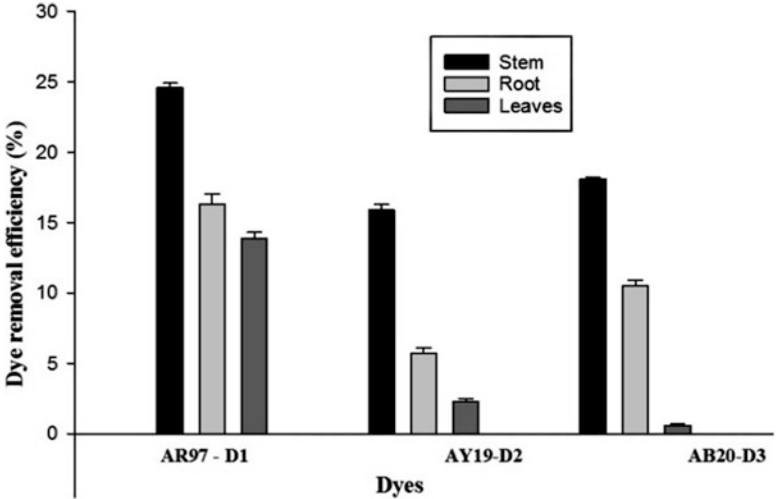

Fig. 3 Effect of plant part on dye removal efficiency for three different dye solutions. (WH dosage = 0.4 g/L, initial dye concentration = 10 mg/L, volume of dye solution = 25 ml, Contact time = 60 min)

Table 6
Effect of plant part on dye removal efficiency for three different dye solutions

Part of WH	Dyes	C_t (mg/L)	Q_t (mg of dye sorbed/mg of WH) $\times 10^{-3}$	R (% of dye removed)
Stem	D_1	7.54 ± 0.03	6.15 ± 0.08	24.6 ± 0.3
	D_2	8.41 ± 0.04	3.97 ± 0.10	15.9 ± 0.4
	D_3	8.19 ± 0.01	4.52 ± 0.02	18.1 ± 0.1
Root	D_1	8.37 ± 0.07	4.07 ± 0.17	16.3 ± 0.7
	D_2	9.43 ± 0.04	1.42 ± 0.10	5.7 ± 0.4
	D_3	8.95 ± 0.04	2.62 ± 0.10	10.5 ± 0.4
Leaves	D_1	8.61 ± 0.04	3.47 ± 0.10	13.9 ± 0.4
	D_2	9.77 ± 0.02	0.57 ± 0.05	2.3 ± 0.2
	D_3	9.94 ± 0.01	0.15 ± 0.13	0.6 ± 0.1

WH dosage = 0.4 g/L, initial dye concentration = 10 mg/L, volume of dye solution = 25 ml, Contact time = 60 min

of cells and this obstructs the dye binding efficiency. Hence, more amount of dye was adsorbed by stem. The maximum amount of AR97 dye ($C_0 = 10$ mg/L) adsorbed by WH stem in 60 min was found to be 6.15×10^{-3} mg of dye/mg of WH. Various dye adsorption processes through different parts of water hyacinth demonstrated that WH stem adsorbed the maximum amount of Acid green dye "200 mg/g" compared to other parts such as root (8.04 mg/g), leaves (20.38 mg/g) [27].

4.4 Effect of Phosphoric Acid Treatment on Dye Removal

The surface phenomena of biosorbent play a major role in the dye adsorption process. The nature of the surface matrix can be modified through physical, chemical, or electrochemical treatments. But greater changes will be resulted from chemical treatment. So far, nitric acid, hydrochloric acid, and hydrogen peroxide had been used to modify the surface chemistry of water hyacinth [28]. Thus in this study an alternative chemical phosphoric acid was studied for its efficiency to increase the porosity and oxidize the Water hyacinth. It was observed that the mean dye removal efficiencies from the triplicate experiment for acid-treated stem for dyes D1, D2, and D3 were 82.54%, 51.37%, and 78.65% respectively. In contrast for untreated stem the values were found to be 24.6%, 15.9%, and 18.1% (Table 7 and Fig. 4). Dye removal efficiency for treated stem increased up to nearly 30–60% and this greater efficiency was achieved in a shorter period of 60 min and sorbent dosage of 0.4 g/L. Thus, it was concluded that greater amount of sorbent mass will further improve the dye removal efficiency.

It was examined that the major reason for improved adsorption of dyes was the activation of carbonization during acid treatment. Thus, the micropores in WH were gradually increased to mesopores and then to macropores and hence this textural change was the major achievement of using phosphoric acid treatment. Comparatively cyanoethylation and amidoximation of WH resulted in

Table 7
Effect of phosphoric acid treatment on dye removal efficiency

WH	Dyes	C_t (mg/L)	Q_t (mg of dye sorbed/mg of WH) $\times 10^{-3}$	R (% of dye removed)
Treated Stem	D_1	1.746 ± 0.12	20.6 ± 0.3	82.54 ± 1.2
	D_2	4.863 ± 0.07	12.8 ± 0.2	51.37 ± 0.7
	D_3	2.135 ± 0.04	19.6 ± 0.1	78.65 ± 0.45
Untreated stem	D_1	7.54 ± 0.09	6.15 ± 0.23	24.6 ± 0.9
	D_2	8.41 ± 0.11	3.97 ± 0.2	15.9 ± 1.1
	D_3	8.19 ± 0.06	4.52 ± 0.15	18.1 ± 0.6

WH dosage = 10 mg/25 ml, initial dye concentration = 10 mg/L, volume of dye solution = 25 mL, plant part used = stem, contact time = 60 min

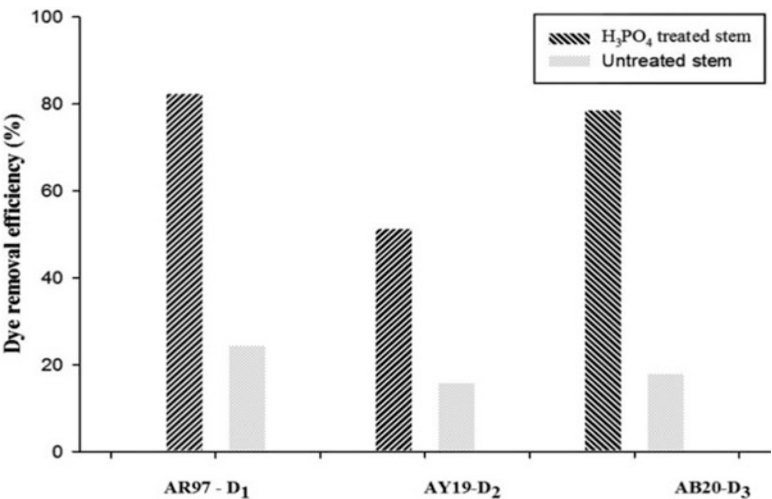

Fig. 4 Effect of phosphoric acid treatment on dye removal efficiency (WH dosage = 0.4 g/L, initial dye concentration = 10 mg/L, volume of dye solution = 25 mL, plant part used = stem, contact time = 60 min)

higher efficiency of dyestuff removal [29]. Previous studies of WH reacted with SDS [30] and charcoal [31] also achieved greater efficiency for the removal of crystal violet and congo red respectively.

4.5 Effect of Initial Dye Concentration

The initial dye concentration study explored that dye concentration is one of the significant factors that poses a considerable variation in dye adsorption efficiency of WH. It was found that as the concentration of the dye molecules increased, collisions between the dye molecules and WH surface increased; this further amplified the number of available binding sites in WH surface to be occupied by dye molecules and thus mass transfer too increased. Increased mass transfer resulted in strong chemical interaction and enhanced dye adsorption. Hence, the increased initial concentration of the dye was an important driving force that raised the mass transfer of three dyes from the aqueous solution onto the surface of PAWH to overcome the boundary layer resistance.

The effect of initial dye concentration of all the three dyes on amount of dye adsorbed for reaction time of 60 min at PAWH dosage of 0.4 g/L was shown in Fig. 5 and their corresponding data were tabulated in Table 8. As the initial dye concentration increased from 5 to 30 mg/L, amount of dye adsorbed for the three dyes AR97, AY19, and AB20 increased from 10.51 to 61.10, 5.87 to 39.75, 1.25 to 6.183 × 10^{-3} mg/mg respectively. Gradual increase in dye concentration gradually increased dye sorption amount till resistance layer exploits its impact on the adsorption. Initially, the dye molecules exist as monomer, as its concentration increased, it forms aggregates and slows down the mass transfer and at some point it reaches equilibrium. In this study, there was no

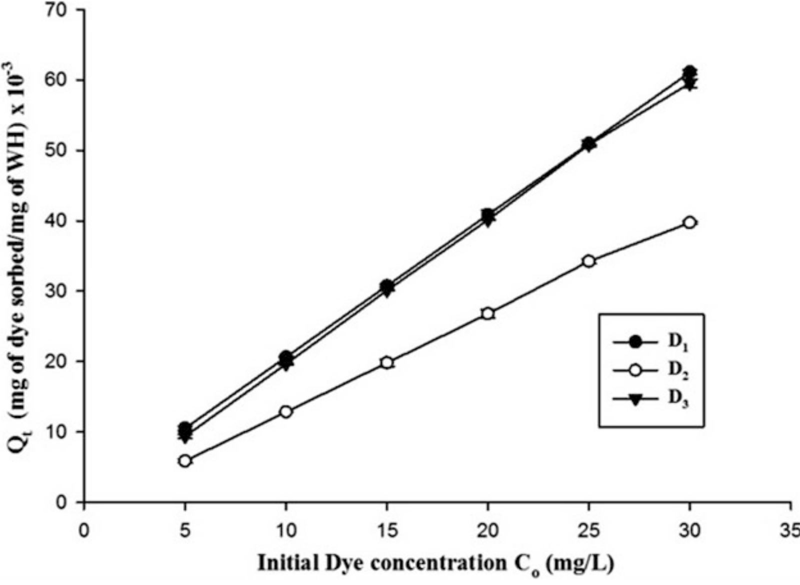

Fig. 5 Effect of initial concentration of three different dye solutions on amount of dye sorbed. (WH dosage = 0.4 g/L, volume of dye solutions = 25 mL, plant part used = H_3PO_4 treated stem, Contact time = 60 min)

Table 8
Effect of initial concentration of three different dye solutions on amount of dye sorbed

C_0 (mg/L)		C_t (mg/L)	Q_t (mg of dye sorbed/mg of WH) $\times 10^{-3}$
5	D_1	0.794 ± 0.15	10.51 ± 0.37
	D_2	2.652 ± 0.12	5.87 ± 0.30
	D_3	1.250 ± 0.09	9.37 ± 0.25
10	D_1	1.746 ± 0.05	20.63 ± 0.12
	D_2	4.863 ± 0.07	12.84 ± 0.02
	D_3	2.135 ± 0.04	19.66 ± 0.10
15	D_1	2.696 ± 0.07	30.76 ± 0.18
	D_2	7.073 ± 0.21	19.81 ± 0.52
	D_3	2.950 ± 0.19	30.12 ± 0.05
20	D_1	3.654 ± 0.25	40.86 ± 0.62
	D_2	9.286 ± 0.23	26.78 ± 0.58
	D_3	3.933 ± 0.07	40.16 ± 0.01
25	D_1	4.607 ± 0.17	50.98 ± 0.43
	D_2	11.30 ± 0.14	34.25 ± 0.35
	D_3	4.675 ± 0.05	50.81 ± 0.01
30	D_1	5.560 ± 0.13	61.10 ± 0.33
	D_2	14.10 ± 0.06	39.75 ± 0.15
	D_3	6.183 ± 0.25	59.54 ± 0.63

WH dosage = 10 mg/25 ml, volume of dye solutions = 25 mL, plant part used = H_3PO_4 treated stem, Contact time = 60 min

equilibrium achieved which explained that PAWH can effectively adsorb the dye molecules >30 mg/L.

Similarly, the previous study of using nitric acid treated water hyacinth stem leaves for adsorption of methylene blue dye showed that as the initial MB concentration increases from 97 to 1187 mg/L, the experimentally observed adsorption capacity, q_{exp}, increases from 48.2 to 333 mg/g. [25]

4.6 Effect of Adsorbent Dosage on Dye Removal

When the concentration of the adsorbent dosage gets increased, the following mechanism takes place:

1. Kinetic energy of dye molecules and PAWH gets amplified.
2. Dye molecules face a greater number of available binding sites and surface area on PAWH, because of increased porosity.
3. This result in increased amount of dye molecules gets adsorbed by PAWH which further results in increase in dye removal percentage.
4. All the above processes occur initially. Later at particular point when all the available outer binding sites are filled with dye molecules resistance develops.
5. Hence, mass transfer slows down and the free molecules have to wait until adsorbed dye molecules have to enter through the PAWH surface. From this point the amount of dye adsorbed reaches a stationary phase and later it reaches equilibrium condition when no more adsorbent is available for dye to get adsorbed.

In this study, it was found that when the concentration of PAWH increased from 0.1 to 1 g/L, dye removal percentage for the three dyes AR97, AY19, AB20 increased from 39.41% to 94.63%, 18.93% to 67.07%, 35.99% to 94.63% respectively and the amount of dye adsorbed increased from 9.85 to 4.6, 4.73 to 16.76, 8.99 to 23.65 $\times$ 10^{-3} mg/mg for the same three dyes (Table 9 and Fig. 6). Further dye removal percentage for the three dyes increased steeply till 0.6 g/L and above this it reached stationary line. The highlight of this experiment is that about 1 g/L of PAWH adsorbs about 98.7 % of dye removal which is the major achievement of this study.

4.7 Biosorption Isotherms

Equilibrium amount of dye adsorbed and equilibrium concentration of residual dye was calculated from the initial dye concentration study. These data were used to find x and y axis variables for adsorption models to plot the isotherms (Table 10). How adsorbate molecules react with adsorbent can be illustrated by adsorption properties and equilibrium data. Series of isotherm models are generally used to express the equilibrium state observed in the adsorption process that is a state of equal distribution of the dye

Table 9
Effect of concentration of H_3PO_4 treated WH stem on dye removal efficiency

Concentration of H_3PO_4 treated WH stem [g/L]	Dyes	C_t (mg/L)	Q_t (mg of dye sorbed/mg of WH) × 10^{-3}	R (% of dye removed)
0.1	D_1	6.059 ± 0.32	9.85 ± 0.80	39.41 ± 3.2
	D_2	8.107 ± 0.14	4.73 ± 0.35	18.93 ± 1.4
	D_3	6.401 ± 0.05	8.99 ± 0.12	35.99 ± 0.5
0.2	D_1	4.437 ± 0.16	13.90 ± 0.40	55.63 ± 1.6
	D_2	6.945 ± 0.06	7.63 ± 0.15	30.55 ± 0.6
	D_3	4.855 ± 0.12	12.86 ± 0.30	51.45 ± 1.2
0.3	D_1	2.906 ± 0.17	17.73 ± 0.42	70.94 ± 1.7
	D_2	5.846 ± 0.06	10.38 ± 0.15	41.54 ± 0.6
	D_3	3.341 ± 0.08	16.64 ± 0.20	66.59 ± 0.8
0.4	D_1	1.746 ± 0.21	20.63 ± 0.52	82.54 ± 2.1
	D_2	4.863 ± 0.41	12.84 ± 1.03	51.37 ± 4.1
	D_3	2.135 ± 0.07	19.66 ± 0.18	78.65 ± 0.7
0.5	D_1	0.747 ± 0.08	23.13 ± 0.20	92.53 ± 0.8
	D_2	4.305 ± 0.15	14.23 ± 0.37	56.95 ± 1.5
	D_3	1.139 ± 0.01	22.15 ± 0.03	88.61 ± 0.1
0.6	D_1	0.258 ± 0.10	24.35 ± 0.25	97.42 ± 1.0
	D_2	3.787 ± 0.20	15.53 ± 0.40	62.13 ± 2.0
	D_3	0.678 ± 0.04	23.30 ± 0.10	93.22 ± 0.4
0.7	D_1	0.179 ± 0.16	24.55 ± 0.40	98.21 ± 1.6
	D_2	3.457 ± 0.25	16.35 ± 0.62	65.43 ± 2.5
	D_3	0.599 ± 0.32	23.50 ± 0.80	94.01 ± 3.2
0.8	D_1	0.135 ± 0.06	24.60 ± 0.09	98.65 ± 0.6
	D_2	3.300 ± 0.19	16.75 ± 0.48	67.00 ± 1.9
	D_3	0.539 ± 0.18	23.60 ± 0.40	94.61 ± 1.8
0.9	D_1	0.130 ± 0.09	24.60 ± 0.15	98.70 ± 0.9
	D_2	3.293 ± 0.19	16.76 ± 0.47	67.07 ± 1.9
	D_3	0.537 ± 0.02	23.65 ± 0.05	94.63 ± 0.2
1.0	D_1	0.130 ± 0.09	24.6 ± 0.15	98.70 ± 0.9
	D_2	3.293 ± 0.19	16.76 ± 0.47	67.07 ± 1.9
	D_3	0.537 ± 0.02	23.65 ± 0.05	94.63 ± 0.2

Initial dye concentration = 10 mg/L, volume of dye solution = 25 mL, plant part used = H_3PO_4 treated stem, Contact time = 60 min

molecules between the liquid phase and the adsorbent. The adsorbent–adsorbate interaction can be predicted from shape of isotherm models [32, 33].

From the isotherm data it was concluded that adsorption capability of PAWH gradually increased with rise in dye concentration and at some point it reached saturation. The Langmuir Freundlich and Temkin isotherm models exploited for experimental data were shown in Fig. 7.

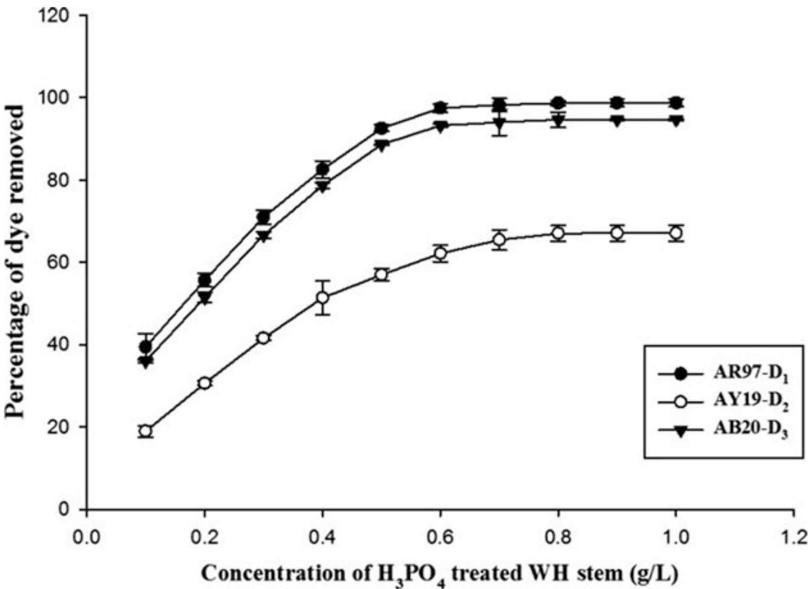

Fig. 6 Effect of concentration of H_3PO_4 treated WH stem on dye removal efficiency (Initial dye concentration = 10 mg/L, volume of dye solution = 25 mL, plant part used = H_3PO_4 treated stem, Contact time = 60 min)

4.8 Langmuir Isotherm

All the four forms of Langmuir isotherm were plotted for the entire initial concentrations of three dyes. From the results it was identified that regression coefficient of Langmuir II model ($R^2 = 0.997$) only related to unity with a minimum deviation. However, R^2 values of other three Langmuir models (I, II, and IV) were the average mean of 0.7. The adsorption process follows a particular isotherm model when the regression coefficient is same or equal to 0.89. Hence, in this study, it was concluded that adsorption of textile dyes by phosphoric acid treated water hyacinth stem follows the Langmuir II isotherm model. One of the Langmuir parameter "$1/Q_m$" indicates the binding affinity of adsorbent and adsorbate. When the $1/Q_m$ value is greater, binding efficiency is also greater [34]. In this experiment, the Q_m (Langmuir monolayer capacity or maximum adsorption capacity) value obtained from the Langmuir II model of the first dye AR97 was smaller and hence its reciprocal value was greater. Thus, the binding efficiency of dye AR97 and PAWH was greater. The smallest observed value of Q_m (212 mg of dye per gram of PAWH) indicated that the dye AR97 formed monolayer coverage around PAWH and this single surface chemisorption was the major rate limiting step.

A similar value of Q_m (112.34 mg/g) was obtained for the previous study of using HCl activated WH for Acid Red dye AR114 [24]. Further analogous results were proved in another study of using water hyacinth stem as adsorbent for AG20 dye where it was concluded that "comparing the correlation coefficient (R^2) values

Table 10
Parameters of Isotherm models determined from Effect of initial dye concentration experiment

C_0		Qe (mg of dye sorbed/mg of WH) $\times 10^{-3}$	Ce (mg/L)	$1/Q_e$ (mg of WH/mg of dye)	$1/C_e$(L/mg)	C_e/Q_e (mg of WH/L)	Q_e/C_e (L/mg of WH) $\times 10^{-3}$	ln Q_e	ln C_e
5	D_1	10.51 ± 0.37	0.79 ± 0.15	95.14 ± 3.23	1.26 ± 0.20	75.16 ± 11.23	13.30 ± 1.73	−4.55 ± 0.03	−0.23 ± 0.17
	D_2	5.87 ± 0.30	2.65 ± 0.12	170.35 ± 8.28	0.38 ± 0.02	451.44 ± 2.49	2.21 ± 0.01	−5.13 ± 0.05	0.97 ± 0.04
	D_3	9.37 ± 0.25	1.25 ± 0.09	106.72 ± 2.77	0.80 ± 0.05	133.40 ± 5.89	7.49 ± 0.32	−4.67 ± 0.03	0.22 ± 0.07
10	D_1	20.63 ± 0.12	1.75 ± 0.05	48.47 ± 0.28	0.57 ± 0.02	84.82 ± 1.92	11.78 ± 0.26	−3.88 ± 0.01	0.55 ± 0.03
	D_2	12.84 ± 0.02	4.86 ± 0.07	77.88 ± 0.12	0.21 ± 0.01	378.50 ± 4.85	2.64 ± 0.04	−4.35 ± 0.00	1.58 ± 0.01
	D_3	19.66 ± 0.10	2.14 ± 0.04	50.86 ± 0.26	0.46 ± 0.00	108.85 ± 1.47	9.18 ± 0.12	−3.92 ± 0.00	0.76 ± 0.01
15	D_1	30.76 ± 0.18	2.70 ± 0.07	32.50 ± 0.18	0.37 ± 0.01	87.77 ± 1.75	11.39 ± 0.22	−3.48 ± 0.01	0.99 ± 0.02
	D_2	19.81 ± 0.52	7.07 ± 0.21	50.47 ± 1.29	0.14 ± 0.01	356.89 ± 1.20	2.80 ± 0.01	−3.92 ± 0.03	1.95 ± 0.03
	D_3	30.12 ± 0.05	2.95 ± 0.19	33.20 ± 0.06	0.34 ± 0.02	97.94 ± 6.13	10.21 ± 0.61	−3.50 ± 0.00	1.08 ± 0.06
20	D_1	40.86 ± 0.62	3.65 ± 0.25	24.47 ± 0.37	0.27 ± 0.01	89.32 ± 4.70	11.19 ± 0.56	−3.19 ± 0.01	1.29 ± 0.05
	D_2	26.78 ± 0.58	9.29 ± 0.23	37.34 ± 0.80	0.11 ± 0.01	346.90 ± 1.05	2.88 ± 0.01	−3.62 ± 0.03	2.22 ± 0.03
	D_3	40.16 ± 0.01	3.93 ± 0.07	24.90 ± 0.01	0.25 ± 0.00	97.85 ± 1.72	10.21 ± 0.17	−3.21 ± 0.00	1.36 ± 0.02
25	D_1	50.98 ± 0.43	4.61 ± 0.17	19.61 ± 0.16	0.22 ± 0.01	90.42 ± 2.55	11.05 ± 0.30	−2.97 ± 0.01	1.52 ± 0.04
	D_2	34.25 ± 0.35	11.30 ± 0.14	29.19 ± 0.25	0.09 ± 0.01	329.92 ± 0.71	3.03 ± 0.01	−3.37 ± 0.01	2.42 ± 0.01
	D_3	50.81 ± 0.01	4.68 ± 0.05	19.68 ± 0.01	0.21 ± 0.00	92.10 ± 0.97	10.85 ± 0.11	−2.97 ± 0.00	1.54 ± 0.01
30	D_1	61.10 ± 0.33	5.56 ± 0.13	16.36 ± 0.09	0.18 ± 0.01	90.99 ± 1.63	10.99 ± 0.20	−2.79 ± 0.01	1.71 ± 0.02
	D_2	39.75 ± 0.15	14.10 ± 0.06	25.15 ± 0.09	0.07 ± 0.00	354.71 ± 0.17	2.81 ± 0.00	−3.22 ± 0.00	2.64 ± 0.01
	D_3	59.54 ± 0.63	6.18 ± 0.25	16.79 ± 0.18	0.16 ± 0.01	103.79 ± 3.07	9.63 ± 0.28	−2.82 ± 0.01	1.82 ± 0.04

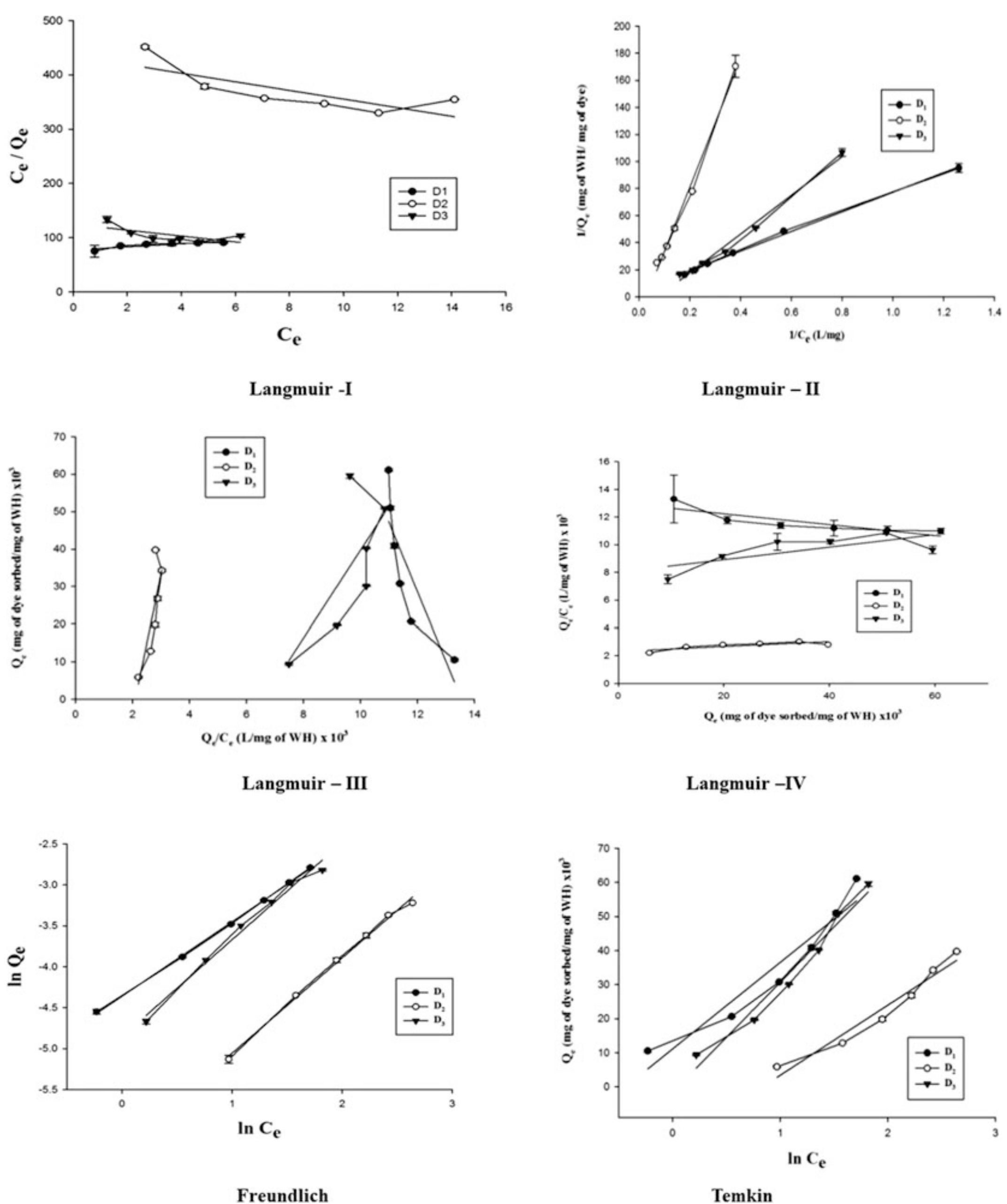

Fig. 7 Isotherm plots

of the four forms of Langmuir and the maximum adsorption capacity, Langmuir-II model ($R^2 = 0.999$) was the most satisfactory model to describe the equilibrium data [19]."

4.9 Freundlich Isotherm

Analyzing the reports of Freundlich isotherm, it was observed that the R^2 values for all the three dyes were relatively higher than Langmuir models. Hence, freundlich isotherm is the much better

isotherm for analysis of data and the goodness of fit goes to the freundlich model. Regression coefficient for this plot was maximum for the first dye ($R^2 = 0.9993$) which was very effective than the Langmuir II model ($R^2 = 0.9975$). Therefore, the Freundlich model was an effective model to fit the experimental data than Langmuir models. In addition, since the slope value of $1/n$ was equal to unity for first dye (0.909) and greater than unity for the other two dyes (1.162 and 1.185), adsorption by PAWH was linear, apt and highly favorable. Since the freundlich model is based on the assumption that there exists a heterogeneous system, in this study it was finalized that surface heterogeneity of PAWH favored multilayer adsorption of three dyes due to the presence of energetically heterogeneous adsorption sites.

Parallel results were received for adsorption of congo red on WH roots and predicted that the Freundlich isotherm model was best applicable for describing the adsorption of CR onto *E. crassipes* roots [35]. Thus, it was ended that PAWH was efficient in the adsorption of dyes from textile dye effluent and followed freundlich isotherm, showing a promising cheap substrate as a potential dye adsorbent.

4.10 Temkin Isotherm

Another isotherm plot for textile dye interaction with PAWH elaborated that the R^2 values (0.9268, 0.9541, and 0.9704) for the three dyes were comparatively lesser than that of the Langmuir and Freundlich models. But the considerable R^2 values have greater impact on the dye sorption mechanism. Thus, the data is better suited by the Temkin Isotherm model also and hence, this model can also be used to determine the adsorption isotherm. Analysis of Temkin constants K_T for the three dyes (1.541, 0.436, and 0.969) showed greater adsorption capacity. Further, the constant B denoting the bonding energy was significant for all the three dyes (0.0254, 0.0205, 0.0323 kJ/mol). Related results were also obtained for adsorption of AG20 dye onto WH stem ($K_T = 1.556$ and $B = 0.285$) [19].

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Chapter 13

Biodegradation of Dyes Intermediates and HPLC Method for Their Estimation

Rana Rashad Mahmood Khan, Saira Saeed, and Ahmad Adnan

Abstract

The industrial synthesis of pigments and dyes generates hazardous chemicals. These chemicals are released into water, soil, and environment and regarded as a major source of environmental pollution. H acid is also an important dye intermediate. In this study, the focus was on the bacterial degradation of H acid (1-amino-8-naphthol-3,6-disulfonic acid) of different concentrations and its degradation estimation method. The biodegradation was identified by the help of IR, UV-Visible spectrophotometer, and high-performance liquid chromatography (HPLC). A method was developed for the estimation of percent biodegradation of H acid with the help of HPLC. The developed method was applicable on standard as well as biodegraded products and on other compounds with the sulfonic acid group. It was found that H acid of concentration 300 ppm and below can be completely degraded with *Bacillus subtilis.* However, over 300 ppm, the bacterial degradation did not work efficiently.

Key words Biodegradation, H Acid, Dyes intermediates, HPLC method, *Bacillus subtilis*

1 Introduction

The modern era is an industrial and technological era; its major concern is with the environmental pollutionBiodegradation. Advancement in chemical industries, agricultural, and other sectors is necessary in fulfilling human population's requirement. All these processes used in the production of goods lead to production of environmental pollution. As a result, these pollutants are released into the air, water, soil and ultimately affect the human health, animals, and as well as plant's growth [1].

In synthetic dyes the most abundant dyes in amount, and numbers are the azo dyes, which are used in different chemical industries [2]. Synthetic dye is difficult to bio-degrade contrasting to natural organic compounds that are readily decomposed or degraded by native microorganisms [3, 4]. Synthetic dyes are toxic in nature and have been highly resistant to degradation because of their complex chemical structure [5].

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Various chemicals intermediates are used for the manufacturing of synthetic dyes among them H acid (1-amino-8-naphthol-3,6-disulfonic acid) is an important intermediate and the basic raw material for the synthesis of chromotrophic acid is H acid [6, 7]. It is used during the synthesis of acid, reactive, azo, and direct dyes. H acid is also used in the corrosion inhibitors, photography and in the production of chromotrophic acid which is dye stuff intermediate. The manufacturing of these dyes involved a number of different stages like sulfonation, nitration, reduction, fusion or hydroxylation, amination, alkylation, oxidation, diazotization, and halogenation in which anthracene, naphthalene, benzene, xylene, and toluene are used as a raw material [8]. The wastes of these industries consist of organic and inorganic material that has high color [9]. Synthetic dyes have wide applications in different sectors, including, textile, lather, chemicals, medicines, food, paper printing and cosmetics, H acid is synthetic dye intermediates. Therefore, it may be present in the effluent of these industrial wastewater [10–13].

Over 7 × 105 metric tons of synthetic dye of 105 different types have been produced annually since 1856 [14]. The synthetic dyes are excessively used in power looms, textile, and other dying and color industries. Among synthetic dyes the contribution of azo dyes is almost 50%. The discharge of these dyes in the effluent is about 10–15%. The major source of liquid effluent pollutants is the textile or dying industry because a huge amount of water is used in dying process [15–17]. About 1 kg of textile material requires 100 L of water for traditional finishing processes [11]. In dying process all dyes did not react with fabric, some of its quantity binds to the fabric from 50 to 98% while other goes through the liquid effluent. The wastewater contains 2% for basic dye and as high as 50% for reactive dye. This waste water contaminates the ground and surface water in its surroundings [18]. According to discreet estimation, every year about 280,000 tons of textile dye are wasted in the form of industrial effluent globally [19]. The industrial textile effluent contains colored substance, which is visually recognizable and color is a first visual identification of contaminates or water is being polluted [20, 21, 14]. Nobody can refuse the importance of color. Therefore, synthetic dyes are used on large scale and their discharge in water is the source of environmental pollution and human health risk [22]. Direct use of industrial dyeing effluent can cause the serious impact on environment and human health [23]. Dyes have the ability to absorb light and therefore, reduce the light penetration power in water bodies' as a result photosynthetic process disturbs, which ultimately affects the aquatic life. The dyes layer at the surface of water decreases the dissolved oxygen and increases the BOD polluted water [24].

Biodegradation is a chemical breakdown of different compounds into various byproducts with the help of various enzymes' actions.

1.1 Biodegradation of Dyes

Biodegradation fragments the synthetic dyes into simpler and smaller parts and also decolorizes. The breakdown of the chromophoric center of dyes results in the form of decolorization of the synthetic dyes [25]. Bacteria, algae, fungi, and yeasts are numerous types of microorganisms used for biodegradation and decolorization of synthetic dyes. These organisms have different capacity for different types of synthetic dyes. Among these organisms, some are preferred over others because of its great capacity for degradation over others. The effective degradation and decolorization depend on the activity of microorganisms selected for that purpose [14]. In the process of biodegradation, favorable conditions and suitable strains are required in the development in the degradation potential [26].

The decolorization and degradation of synthetic dye with the help of bacteria is normally fast than other microorganisms [15]. Azo dyes are degraded into aromatic amines under anaerobic conditions with the help of bacteria while aromatic amines do not degrade under anaerobic conditions while readily available under aerobic conditions [27]. Herein, special consideration was paid to the aerobic degradation of sulfonated azo dyes intermediates because it is used as a raw material in the synthesis of sulfonated azo dyes [28].

2 Materials

The biodegradation of H acid was performed with *Bacillus subtilis*. The structure of H acid is given in Fig. 1. The experimental procedure is detailed below.

2.1 Broth Media Preparation

4.5 g of nutrient broth was taken and dissolved in 300 ml of distilled water in a flask. The flask was cotton plugged and sterilized in an autoclave at 121 °C for 15 min. After the completion of sterilization, the nutrient broth was transferred into six flasks each containing 40 ml and cotton plugged. The transfer of nutrient broth was carried out in a laminar air flow to avoid contaminations.

NH2 OH
HO S O O
S OH O O
1-amino-8-naphthol-3,6-disulfonic acid)H acid)

Fig. 1 Structure of H acid

2.2 Inoculum Preparation

1.5 g of nutrient broth was taken and dissolved in a 100 ml of distilled water and sterilized well. The flask was taken in an incubator and suspension of *Bacillus subtilis* transferred into the nutrient media and cotton plugged. The flask was placed in an incubator shaker for 24 h to grow the inoculum media. After 24 h, 1 ml of inoculum was added in each flask containing 40 ml of nutrient broth and kept in an incubator for 24 h for the growth of bacteria.

The biodegradation of H acid was performed at pH 3–4 in 250 ml Erlenmeyer flask in which different weights of H acid and 40 ml of nutrient media were taken after bacterial growth. The samples of different concentrations from 100 ppm to 1000 ppm were prepared and analyzed for their biodegradation at different time intervals. The collected samples were first analyzed by taking their absorbance through UV-Visible spectrophotometer [UV 1800, Bruker, model (S/N) (A11454804935)]. The further degradation was confirmed by different analytical techniques such as IR, HPLC, and UV spectrophotometer. The HPLC [Shimadzu Lab Solutions, mode: LC, Detector: UV 254 nm, Column: Agilent Zorbax-CN (4.6-mm × 250-cm), 5 μm] chromatogram was also recoded and the RP-HPLC method was developed for their analysis.

2.3 Mobile Phase Composition and Chromatic Conditions

Methanol to Triethylamine buffer ratio was 1:10. Dilute 4 ml of triethylamine with a sufficient amount of water was used to produce 1000 ml of buffer solution. The pH 2.7 of the solution was adjusted with phosphoric acid (85%) and filtered with 0.45 μm filter. The chromatographic conditions employed in the given conditions are:

Column: Agilent Zorbax-CN (4.6-mm × 250 cm), 5 μm, Temperature: 25 °C.

Flow rate: 1 ml/min.

Detection wavelength: 218 nm.

Injection volume: 10 μl.

Retention time: 10 min.

3 Methods and Results

A method was developed in H acid analysis, and the validated results are shown in Table 1.

Standard and specimen of H acid were prepared, and the % Relative Standard Deviation (% RSD) was calculated against retention time and peak area under the standard. The specimen was prepared (six times) in replicate to analyze and conclude the components and % RSD of components. As per given data in Table 1, the % RSD for H acid is 0.180; retention time is 0.016 and assay content is 0.47, which fall within the acceptable standards of precision; the method is precise.

Table 1
Validation resultsValidation parameters

Validation Parameters	H acid
Specificity	Specific
Peak purity index	1.000
Precision (repeatability)	
System (%RSD)	99.25 ± 0.18
Method (%RSD)	99.25 ± 0.47
Precision (reproducibility)	
Intra-day* (% RSD)	99.25 ± 0.47
Inter-day* (% RSD)	99.72 ± 0.50
Accuracy	
Level I ($n = 3$) (% RSD)	99.53 ± 0.13
Level II ($n = 3$) (% RSD)	99.81 ± 0.11
Level III ($n = 3$) (% RSD)	99.57 ± 0.18
Linearity range (μg/ml)	80–120
r [2]	1
% Y-intercept	−0.04
Slope	4.42504×10^4
Robustness (cumulative %RSD)	
Change in flow rate	
0.8 ml/min ($n = 3$)	99.66 ± 0.34
1.2 ml/min ($n = 3$)	99.59 ± 0.37
Change in detection wavelength	
216 nm ($n = 3$)	99.63 ± 0.39
220 nm ($n = 3$)	99.78 ± 0.37
Change in column temperature	
23°C ($n = 3$)	100.07 ± 0.42
27°C ($n = 3$)	99.35 ± 0.42
Change of column	
Merck col1 ($n = 3$)	99.74 ± 0.33
Teknokroma col. 2 ($n = 3$)	99.80 ± 0.36
LOD (μg/ml)	0.351
LOQ (μg/ml)	1.063
Analytical solution stability (cumulative % RSD)	0.427

The intermediate precision also lies in the standard range, for recovery experimentation standard addition method was implemented. The three levels of accuracy established for these solutions were 80%, 100%, and 120%. The observed results are within the acceptance criteria; the method is accurate. The data are given in Table 1.

The five levels of linearity at different concentrations, i.e., 80, 90, 100, 110, and 120 ppm were performed and the data given in Table 1 show that measured values lie within the acceptance criteria, hence the method is linear.

The limit of detection (LOD) and limit of quantitation (LOQ) for the method validation of H acid are derived from the residual standard deviation of the regression line based upon the detection limit co-efficient, and quantitation limit co-efficient 10. The data in the Table shows that the method is applicable to the detection and quantitation of H acid. The LOD and LOQ results are given in Table 1.

The analytical solution stability was measured by performing the analysis of the sample solution at different time intervals. The prepared solution is kept for 24 h in a lab and then analyzed; the results were compared with freshly prepared solution, and robustness (I-VIII) in which the change in mobile phase flow rate, wavelength, HPLC column of different series, and temperature was examined and the obtained results fall within the acceptable range, maintained by ICH, confirming the validity of method for the analysis of chemical intermediates and their degradation products.

The H acid is an important dye intermediate, which is synthesized by following the sulfonation, nitration, reduction, and alkali fusion/hydroxylation. It is widely used during the synthesis of direct, azo, and reactive dyes. The dyes prepared from H acid are toxic and carcinogenic in nature. They also cause environmental and aquatic pollution, and therefore, their degradation is a hot issue for the researchers working in similar areas. Herein, biodegradation of H acid was performed with *Bacillus subtilis*. The UV-Visible spectrophotometer of H acid in Fig. 1 depicted three absorption bands at 338.8, 239.2, and 220.4 nm. During the degradation study, the absorption band at 338.2 nm was fully vanished after short intervals of time with respect to the concentration of H acid. The 338.2 nm band was completely disappeared in 100 ppm concentration after 1 h and the lower intensities were observed for other concentrations. The other two bands in 100 ppm concentration totally vanished after 12 h of incubation at 37 °C. While in higher concentrations, these bands took longer times to disappear. The tentative pathway of H acid degradation is given below in Fig. 2, [16–29]. The aim of this study was to develop an accurate, sensitive, rapid, reliable, validate, and an adequate method for the analysis of organic compounds and chemical intermediates

Fig. 2 Tentative pathway of H acid degradation

especially for H acid and its degradation products that are applicable in the routine laboratory analysis. A method was developed for the estimation of H acid. This method can be applied to other organic compounds containing amino and sulfonic acid groups and their degradation products. The validation results are given in Table 1.

All the parameters were within the acceptance criteria in the validation of method. The same method was applied to the biodegradation products and the percentage degradation of H acid with the passage of time was calculated.

3.1 Identification of Biodegradation of H Acid

The biodegradation of H acid was identified by the help of IR, UV-visible spectrophotometer (Fig. 3) and high-performance liquid chromatography. For taking the IR spectrum, in FTIR a sample is placed on the port and scanned. It requires no disc preparation in Bruker Optics. The same procedure was adopted for sample and reference. The IR spectrum of H acid standard and biodegradation products are given in the following figures. Each peak represents different functional groups that may be expected to be present the specimen. The peaks with reference to their functional groups are

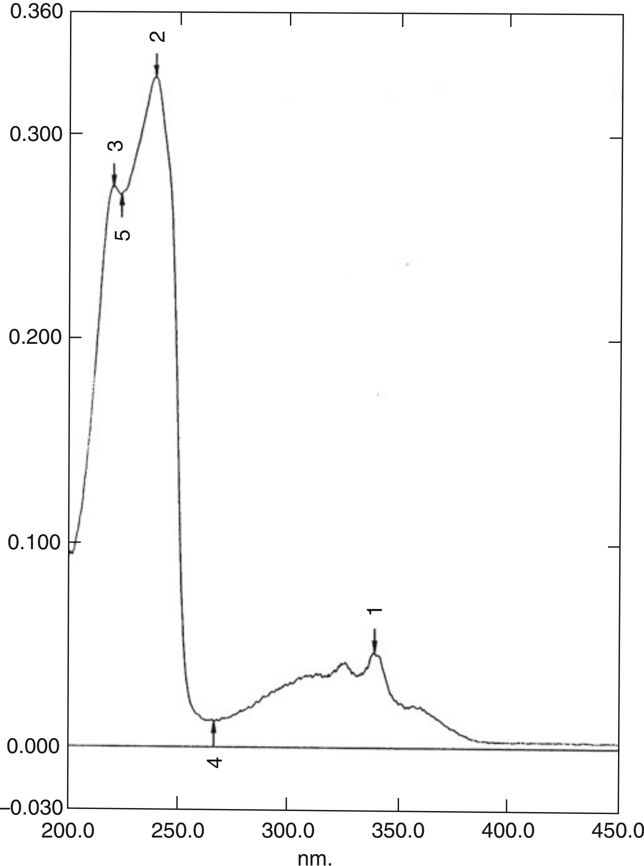

Fig. 3 UV absorption spectrum of H acid

given by Fig. 4. IR (KBr, cm^{-1}) \upsilon$_{max}$ 3500(N–H), 3310 (O–H), 2878(C–H), 1597 (C=C), 1370(S=O) and Fig. 5. IR (KBr, cm^{-1}) \upsilon$_{max}$ 3287 (O-H), 1638 (C=C).

The peaks of IR spectrum show the biodegradation of H acid. There are only two peaks on the spectrum after its degradation at 3287 (O-H), 1638 (C=C) which indicates the presence of O-H and C=C bonds (Fig. 4). The disappearance of other peaks on the spectrum indicates the degradation and appearance of peaks at different positions designates the formation of other intermediate products.

The biodegradation was also identified by the help of HPLC. The change in peak's retention times in HPLC chromatogram shows the degradation of H acid. The peak at retention time 5.58 min indicates the H acid while in the lower chromatogram in Fig. 6, the absence of main peak at 5.58 min indicates the degradation of H acid and appearance of new peak at 3.2 min verifies the formation of other intermediates during the degradation process.

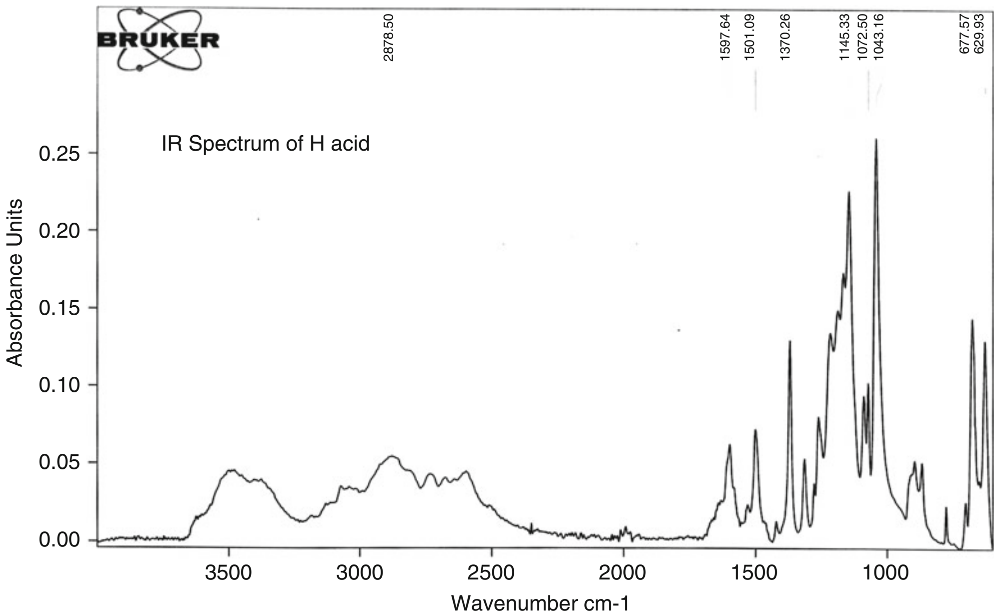

Fig. 4 IR spectrum of standard H acid

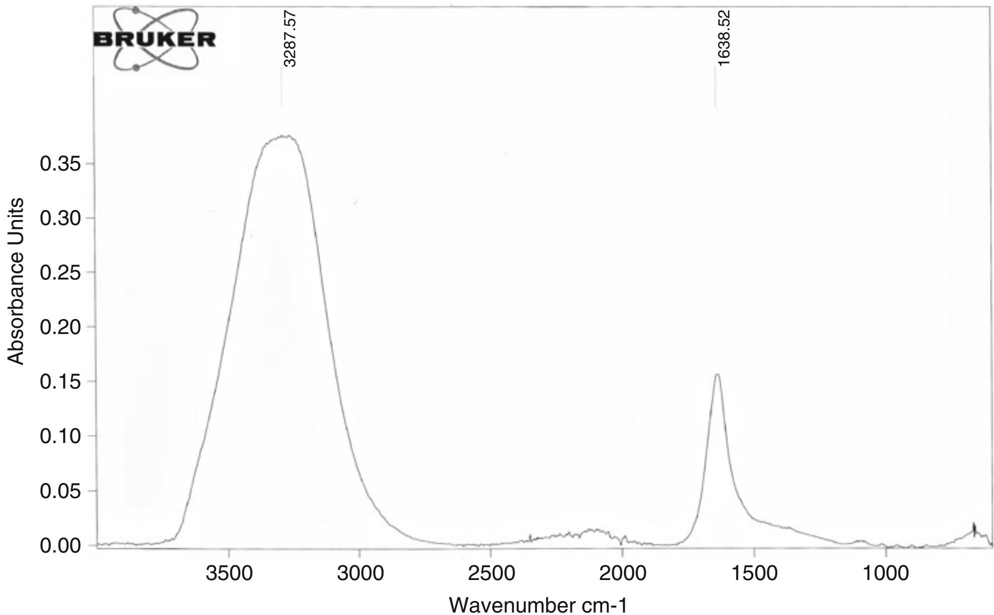

Fig. 5 IR spectrum of H acid after biodegradation

Figure 7 shows the biodegradation of H acid at different concentrations. The graph in Fig. 8 shows the percent biodegradation of H acid. The H acid of lower concentration, i.e., 100 ppm was completely degraded during the course of 12 h. The biodegradation of H acid of 200 ppm was completed during the course of

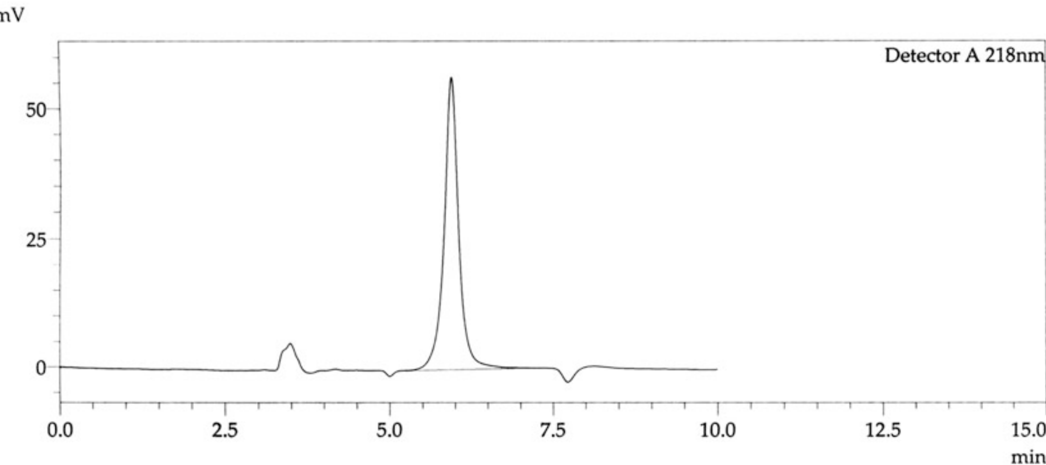

Fig. 6 Chromatogram of H acid (5.58 min)

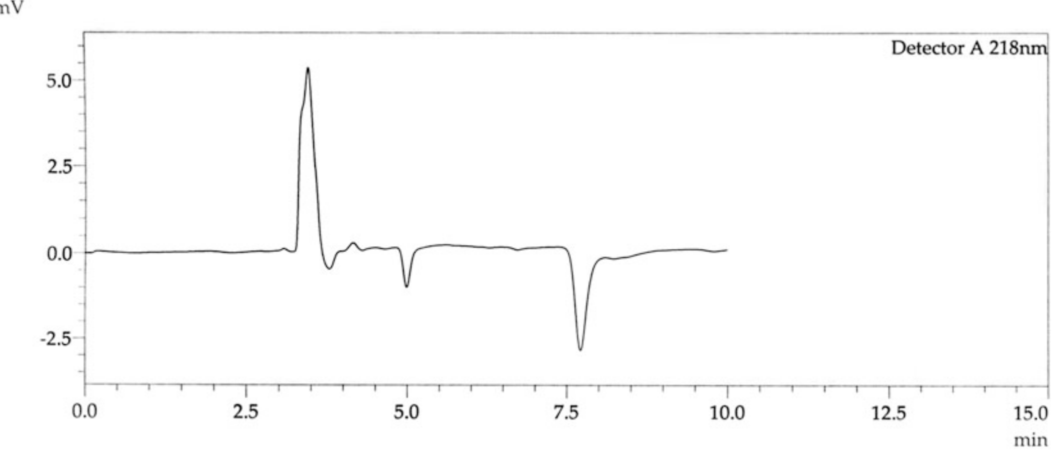

Fig. 7 Chromatogram of H acid after biodegradation

24 h. The higher concentrations over 200 ppm take longer time for degradation under normal conditions and do not completely degrade. The higher concentrations above 200 ppm were incubated for 48 h, but their degradation was not completed in this course of time. The H acid of 300 and 400 ppm concentration was degraded up to 94% and 34% respectively and 500 ppm was degraded up to 20% in the course of 48 h with an interval. There was no degradation found over 500 ppm. The complete biodegradation up to 200 ppm is possible with *Bacillus subtilis*. The concentration has an adverse effect on degradation. The lower concentration was completely degraded with less time while the

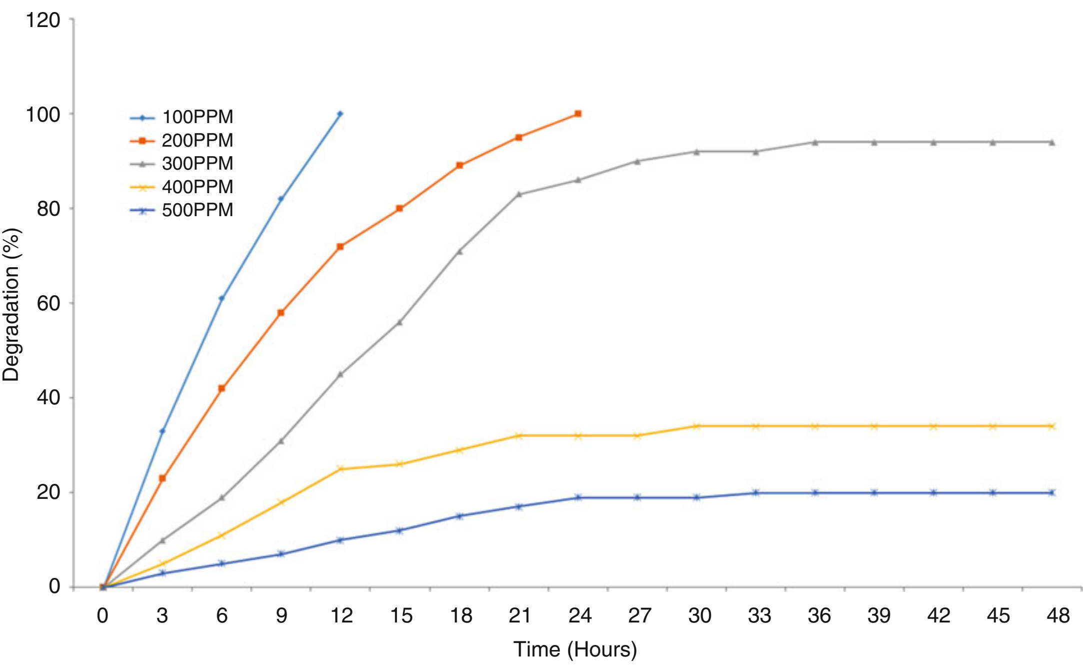

Fig. 8 Percent biodegradation of different concentrations of H Acid with time in *Bacillus* sp

solutions of higher concentrations are difficult to degrade and take longer time for their biodegradation. The lower concentration was completely degraded with less time while the solutions of higher concentrations are difficult to degrade and take longer time for their biodegradation.

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Chapter 14

Biodegradation Behavior of Textiles Impregnated with Ag and TiO_2 Nanoparticles in Soil

Maja Radetić and Zoran Šaponjić

Abstract

Increasing global consumption of textile goods poses serious environmental problems that are present throughout the products life-cycles. With a trend of Fast Fashion, clothes became consuming goods that are discarded in huge amounts into landfills where they rot generating undesirable gases which contribute to environmental pollution and greenhouse effect. A large-scale centralized composting is a possible alternative to harmful landfilling. It is well known that different textile fibers exhibit diverse biodegradation behavior. Unlike most of the synthetic fibers, natural and particularly cellulosic fibers are prone to biodegradation. However, the finishing and thus, the presence of different chemicals on fiber surfaces may affect their biodegradation performance. Recent progress in the production of textiles impregnated with different metal and metal oxide nanoparticles is more oriented toward end-use achievements than on their environmental and health safety impacts. Despite the urgency to establish their environmental risk assessment, there are only few papers dealing with biodegradation behavior of such textile nanocomposites under terrestrial conditions. Therefore, this chapter is aimed to provide an insight into these results and to stress the necessity of extended research in this field taking into consideration that various characteristics of nanoparticles are relevant for their biodegradation behavior.

Key words Biodegradation, Textiles, Ag nanoparticles, TiO_2 nanoparticles, Soil

1 Trends in Textile Solid Waste Management

Global fiber and clothing production has been continuously growing over the past several decades. The increase in fiber demand and consumption is a consequence of global population growth and overall improvement in living standards [1]. At the same time, the generation of textile solid waste was rapidly increasing. Challenging problems dealing with a management of textile solid wastes can be illustrated by the most recently released figures from the EPA: the United States of America alone generated about 15.13 million tons of textile waste in 2013, accounting for around 6 wt% of the total municipal solid waste at 254.11 million tons per year [2]. According to this report, only 15.2 wt% of textile waste was recovered and 84.8 wt% was discarded. Conventional methods for management of

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textile solid wastes are landfilling, incineration, and recycling [3]. Although recycling of textiles is the most preferable approach, the landfilling and incineration are considerably more practiced. In addition to green-house gasses emissions, the major disadvantage of landfilling is tipping fee [1]. Introduction of taxes on landfills resulted in illegal waste dumping in many countries. Such a practice has adverse effects on local environment, i.e., it can pollute land and local waterways, and can pose a health risk to the community.

In terms of biodegradable textile solid waste management, a large-scale centralized composting facility is viewed as a viable alternative to landfilling and incineration [3]. So far a small amount of textile waste was evaluated in large-scale composting streams despite good laboratory results on their biodegradability [3–5]. Generally, biodegradability of textile materials is a very important issue for waste management and it is often recognized as an indicator of environmental friendliness of the material [4]. However, different chemicals that are used for impregnation of textile fibers during the finishing might have negative influence on the biodegradation process. Taking into consideration the increasing growth of textile solid waste and advanced finishes that are imparting specific end-use properties, it is essential to assess their influence on biodegradability of textile fibers. New generation of nano-finish formulations particularly require urgent evaluation.

2 Environmental Aspect of Textile Nano-finishing

There is almost no sector in industry today where nanotechnologies are not more or less interfered. Textile industry promptly recognized the potentials of nanotechnologies and growing applications exceeded an initial idea of their exploitation for technical textiles. Nanotechnologies in textiles could be divided into three broad categories: fabrication of nanofibers by electrospinning, surface modification of fibers and fabrics with various nanoparticles (NPs) known as nano-finishing, and fabrication of nanoparticle composite fibers. Coating of textile surfaces with different NPs is often seen as a promising alternative to conventional finishing processes which can impart antimicrobial, UV protective, self-cleaning, antistatic, flame retardant, wrinkle resistant, water and oil repellent properties to textile goods [6, 7]. Major research so far was focused on functionalization of textile surfaces with Ag NPs (antimicrobial activity) and metal oxide NPs with high photocatalytic activity such as TiO_2 and ZnO NPs (antimicrobial activity, UV blocking, and self-cleaning properties) [6–17]. The advantages of textile nano-finishing rely on relatively easy routes for the synthesis of different NPs and recent availability of commercial NPs, the fact that small amount of NPs can provide desired effects as well as that chemical formulations and application technologies could be

relatively easily adopted by existing textile mills. However, the key drawback of textile nano-finishing that prevents broader commercialization of nano-engineered textile goods is relatively poor durability of generated effects particularly during washing. Consequently, different approaches have been developed that may enhance binding efficiency of NPs such as plasma activation of textile materials, the use of spacers, in situ fabrication of NPs on textile surfaces, layer-by-layer deposition, etc. [14, 18–29].

Broad research in this field was mainly oriented toward obtaining desired effects and development of suitable approaches to achieve them. Despite urgent need for environmental and health safety risk assessment because of the relatively low durability of metal and metal oxide NPs incorporated into textile materials, their environmental impact was not sufficiently explored. Accidental or intended discharge of engineered NPs from textile goods into natural environment is expected to occur throughout their life cycles: from their production to exploitation and disposal [30–33].

Since Ag NPs are exploited for impregnation of textile materials to a large extent, the growing interest in environmental performance of these NPs is not surprising. Keeping in mind that the processes of impregnation are usually wet processes as well as that textile goods are frequently washed during their exploitation, it is reasonable that the major work was focused on behavior of textile nanocomposites particularly with Ag NPs in aquatic environment. Hence, large share of textiles in Ag emissions into water lately attracted more scientific attention [34–36]. A vast research on Ag NPs release from textiles into water is a fundamental need for development of models that would predict the probability distributions of mass flows and exposure concentrations in water, soil, sludge, air, etc. [37–40].

A knowledge on their fate in terrestrial environment, i.e., their possible toxicity and influence on biodegradation of textile materials in soil is limited at this point. There are at least two reasons for relatively slow progress in environmental risk assessment: variety of NPs and the lacking of adequate ecotoxicological and analytical testing methods which can detect, quantify, and characterize NPs under ambient conditions [30]. The characteristics of NPs such as size, shape, chemical composition, surface structure, charge, solubility, and aggregation state seem to be very important for their environmental impact and all of them must be adequately judged [41]. The presence of frequently used stabilizers (citrate, cysteine, polyethylene glycol or starch, etc.) may also interfere and considerably alter the NPs interaction with environment. On the other hand, the properties and thus, behavior of material in the form of NPs may remarkably differ from the bulk material [30]. Therefore, existing testing methods developed for bulk materials cannot be simply transferred to materials in nano regime. Accordingly,

environmental risk assessment originating from NPs obviously will not be an easy task as general conclusions valid to all products containing NPs could not be drawn.

3 Biodegradation Testing Methods of Textiles

Biodegradability of textile materials under laboratory conditions is commonly tested by the soil burial test, ASTM D 5988-03 standard method (Standard test method for determining aerobic biodegradation in soil of plastic materials or residual plastic materials after composting) [42] based on monitoring of CO_2 as a product of microbial mineralization and enzymatic hydrolysis [3–5, 43–51]. A variety of physical and chemical methods are currently available to assess the changes produced by microbial metabolism of a substrate [3, 4, 43]. If the substrate is large enough and insoluble in water such as textile fibers, physical measurements are normally applied for the evaluation of the initial microbial attack of the substrate [43]. Usually, microscopic examination (optical microscopy), measurement of the weight loss, and measurement of the loss of mechanical strength are included. Microbial depolymerization of macromolecules due to hydrolysis of chemical bonds weakens the fibers and hence, examination of mechanical properties provides valuable data [43]. In order to follow the biodegradation progress and to obtain complete characterization of the investigated textile material, the combination of some of these methods is generally involved in a single study [3–5, 44–52]. Macroscopic changes of the fabric are often verified by camera while the change of the color of the fabric due to rotting is measured spectrophotometrically. For the detection of delicate changes in the structure/chemical composition of fibers and their morphology, Fourier transformed infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) are applied, respectively. SEM is also used for the detection of microbial colonization on the fibers [43]. Park et al. used X-ray diffraction (XRD) for the evaluation of changes in cotton fiber crystallinity [4].

4 Biodegradation Testing of Cotton and Cotton/PET Blends Impregnated with Metal and Metal Oxide Nanoparticles in Soil

Although it is generally accepted that characteristics of NPs determine their behavior in environment, recent reports underlined the relevance of these features during the biodegradation of textile in soil and need for extended research in this field [46–51]. The first attempts to elucidate the influence of chemical structure, form, and size of Ag NPs on biodegradation of cellulose fibers were made by

Klemenčič et al. [46]. Their study relied on chemical modification of cotton fabrics with three commercially available products based on silver in different forms: nanopowder of silver with an average particle size of 30 nm, a dispersion of AgCl and colloidal Ag NPs with an average particle size smaller than 10 nm. This research pointed out that the soil burial test could be a good choice for the evaluation of biodegradation behavior of such textile nanocomposites. 3 and 12 days long tests were carried out in commercial grade compost. As expected, visual examination showed that the largest fabric damage appeared on the control, untreated cotton fabric. The samples turned to brownish color due to rotting. Spectrophotometric measurements demonstrated that the samples impregnated with powdered Ag NPs only slightly inhibited fiber biodegradation even when higher concentrations of dispersion were applied. In contrast, the samples impregnated with AgCl particles and particularly colloidal Ag NPs protected the fabrics from biodegradation. The color of the latter sample was insignificantly changed, this being in line with SEM analysis which revealed that these fibers were almost unaffected by soil microorganisms.

Taking into account that mechanical properties of textile materials are directly affected by the degree of sample degradation, the breaking strength and the degree of polymerization were determined [46]. Mechanical properties were expressed via relative reduction of breaking strength (q_{red}) of buried samples compared to unburied once. The results shown in Fig. 1 confirmed that the mechanical properties of the samples impregnated with AgCl particles and colloidal Ag NPs did not significantly decrease during exposure to soil microorganisms [46]. At the same time, the degree of cellulose polymerization decreased with burial time and it depended on the form of applied silver and concentration. The largest drop was detected after 12 days of burial in the untreated sample (around 15%) and the lowest in the samples impregnated with AgCl particles and colloidal Ag NPs (less than 1.5%).

This research also confirmed the previous results reported by Tomšič et al. that the progress of biodegradation can be evaluated by FTIR analysis [45]. Namely, it was established that the appearance of bands at 1640 and 1548 cm^{-1} corresponding to amides I and II groups originates from protein production during microbial growth on fibers [46]. In other words, the larger the biodegradation degree, the larger the microbial growth and hence, the intensities of the bands at mentioned wave numbers. Accordingly, the intensity of these bands increased to largest extent in the untreated sample after 12 days of burial, followed by the samples impregnated with powdered Ag nanoparticles and finally with AgCl and colloidal NPs.

The authors found an explanation for diverse behavior of differently finished cotton fabrics in the chemical structure and the size of Ag NPs [46]. Taking into account that both Ag NPs and Ag^{+}-ions exhibit strong antimicrobial activity as well as that release

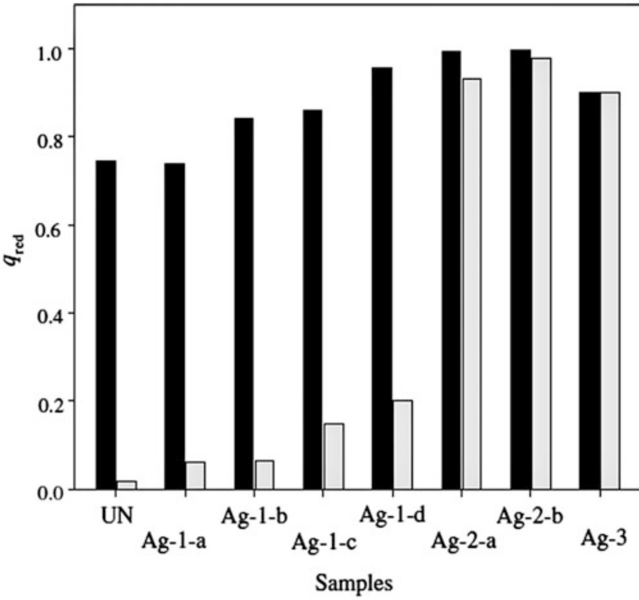

Fig. 1 The loss of breaking strength, $q_{red,t}$, of cotton samples after 3 (*filled square*) and 12 (*open square*) days of soil burial. *UN* unfinished sample (Ag-1-a: Ag nano powder, 0.05%; Ag-1-b: Ag nano powder, 0.10%; Ag-1-c: Ag nano powder, 0.25%; Ag-1-d: Ag nano powder, 0.50%; Ag-2-a: AgCl particles, 0.15%; Ag-2-b: AgCl particles, 0.30%; Ag-3: colloid, 0.05%). Reproduced from [46]

of Ag^+-ions can occur from Ag NPs as a result of oxidation in the presence of oxygen and water [53, 54], it was assumed that the antimicrobial activity of the finish based on the powdered Ag NPs was only influenced by the release of Ag^+-ions from Ag NPs agglomerated on the fabric surface. In fact, they believed that the rate of Ag^+-ions release was too slow and the concentration of the generated Ag^+-ions was below the critical concentration of biodegradation inhibition. Despite the similar degree of agglomeration, AgCl particles provided much better inhibition of biodegradation likely due to different mechanisms of Ag^+-ions release which relied on dissociation of the salt in water. It was supposed that in this case sufficient amount of Ag^+-ions has been produced. Eventually, the strongest biodegradation inhibition of cotton fabric impregnated with colloidal Ag NPs was attributed to synergetic biocidal activity of both Ag NPs and Ag^+-ions. They suggested that large surface area of small Ag NPs enabled high rate of Ag^+-ions release from the particle surface which resulted in increase of antimicrobial protection.

The same group of authors expended their research to biodegradation behavior of cotton/PET blend in soil [47]. Both cotton and cotton/PET fabrics were impregnated with commercial colloid consisting of Ag NPs with an average size smaller than 10 nm. This research confirmed that combination of visual evaluation, spectrophotometric color measurement, SEM analysis of fiber

morphological changes, determination of the loss of breaking strength, and FTIR analysis of induced chemical changes seem to be a feasible and efficient combination of methods for the evaluation of biodegradation behavior of both fabrics. As supposed, the larger the concentration of applied colloid, the stronger the inhibition of biodegradation. Biodegradation was more prominent on the blend due to smaller amount of the deposited Ag NPs. Mechanical properties were preserved only when higher concentration colloids of Ag NPs were used. FTIR analysis revealed that the spectra of cotton samples impregnated with larger amount of Ag NPs were not significantly changed after 12 days of soil burial test. Unlike them, in the spectrum of insufficiently protected cotton fabric a decrease in intensity of absorption bands associated with decrease in crystallinity of degraded fibers occurred [47]. This is in line with findings of Park et al. [4]. Their XRD measurements indicated that crystallinity initially increases since the soil microorganisms attack amorphous regions in the first place [4]. With a progress of biodegradation process, the crystallinity decreases.

This research made an important contribution to understanding of chemical changes in the cotton/PET blend during the biodegradation by FTIR [47]. Namely, they showed that the PET component of the control blend remained almost undamaged after 12 days of burial. It was concluded that an increase in the intensity of the band centered at 1720 cm^{-1} should be ascribed to both carbonyl stretching vibrations of PET and carboxyl/aldehyde groups originating from the oxidative degradation of cellulose during enzymatic degradation, i.e., opening of the β-D-glucopyranose rings [47]. In addition, the biodegradation of cellulose component in the blend resulted in decrease of the band at 3340 cm^{-1} which is related to surface hydroxyl groups. Like in the former study, the bands corresponding to proteins of microorganisms could be detected. In contrast to control blend, Ag NPs hindered rotting of the fabric. However, it was evident that the intensity of the absorption bands at 1640 cm^{-1} and 1548 cm^{-1} corresponding to amides I and II groups gradually decreased as the Ag NPs concentration on the fiber increased. Consequently, this band completely disappeared from the spectrum of the blend impregnated with the largest amount of Ag NPs (Fig. 2).

Ibrahim and Hassan proposed a green route for the fabrication of Ag NPs based on the reduction of Ag^{+}-ions using the biomass filtrate of fungus *Alternaria alternata* [48]. Cotton fabrics were impregnated with synthetized Ag NPs by the dip-coating method from the solution containing Ag NPs and binder butyl acrylate. The biodegradation progress was checked after 14 days of soil burial test. Unlike in the case of untreated cotton fabric where visual color changes due to rotting were obvious, the color of the samples impregnated with Ag NPs from the colloids of different concentrations was not changed indicating the suppression of the

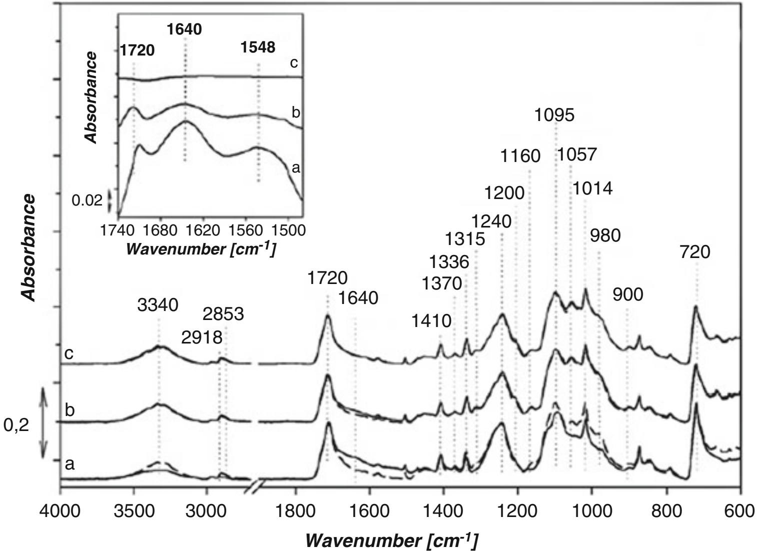

Fig. 2 IR ATR spectra of CO/PET fabrics finished by agents Ag before (–) and after 12 days of soil burial (-). Samples: (**a**) 0.005 (o.w.f)%, (**b**) 0.025 (o.w.f)%, and (**c**) 0.05 (o.w.f)% of Ag agent. *Inset*: differential spectra obtained by subtracting spectrum of the sample before soil burial from spectrum of the sample after 12 days of soil burial in the 1740-1480 cm^{-1} spectral region. Reproduced from [47]

biodegradation. These observations were in alignment with the results of the tensile strength and the elongation at break measurements. As could be predicted, the tensile strength and the elongation at break of untreated samples considerably decreased after 14 days of soil burial test. Mechanical properties of cotton fabrics impregnated with Ag NPs were not significantly changed which was attributed to antimicrobial protection provided by Ag NPs.

Presented studies confirm that the evaluation of textile mechanical properties seems to be a viable indicator of biodegradation progress. However, such measurements require larger samples, i.e., larger soil surfaces/volumes for burial testing. Such an experimental setup is not always possible to provide under laboratory conditions. Consequently, instead of mechanical properties, weight loss of samples could be measured [3, 49, 50, 52]. In this case, the size of sample is not strictly defined.

Lazić et al. compared the influence of Ag and TiO_2 NPs on biodegradation behavior of cotton fabrics using standard test method ASTM 5988-03 based on the determination of percentage conversions of carbon content to CO_2 and soil burial test where the extent of biodegradation was estimated by the calculation of fabric weight loss [49]. This research also demonstrated that the results of hydrolysis of cotton with enzyme cellulase are in good correlation with mentioned biodegradation tests. As expected, Ag NPs hindered the biodegradation process due to extraordinary antimicrobial activity. Taking into consideration that TiO_2 NPs exhibit

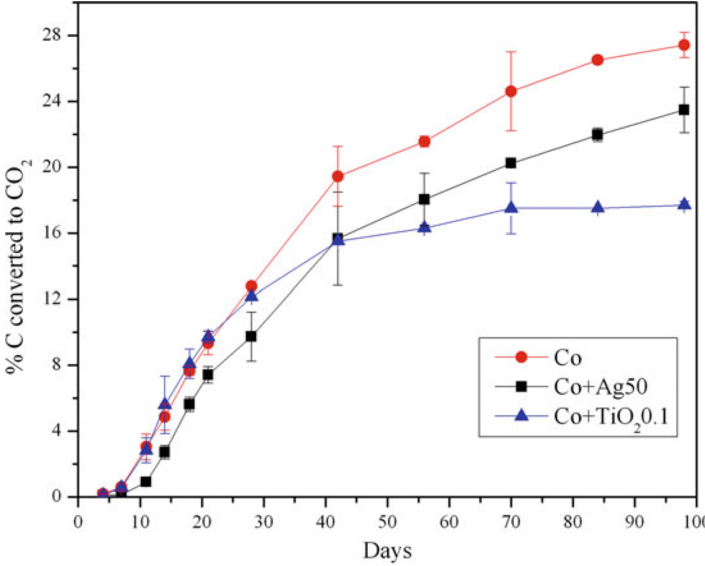

Fig. 3 Biodegradation of control cotton fabric (Co), cotton fabric impregnated with Ag NPs from 50 ppm colloid (Co + Ag50) and cotton fabric impregnated with TiO_2 NPs from 0.1 M dispersion (Co + $TiO_2$0.1) tested in accordance with ASTM D 5988-03 at average temperature ~25 °C. Reproduced from [49]

antimicrobial activity only when they are exposed to UV light, it was not anticipated that they would hinder the biodegradation process even stronger than Ag NPs (Fig. 3). This study also revealed that the larger the content of both NPs, the stronger the biodegradation inhibition. In addition, temperature has strong influence on biodegradation rate.

Unexpected adverse influence of TiO_2 NPs on biodegradation of cotton was assumed to be due to uniform distribution of TiO_2 NPs over the surface of cotton fabric confirmed by the XPS analysis which made the surface more hydrophobic [29]. Such an assumption is based on research of Priester et al. who showed that since microorganisms in soil are primarily associated with soil organic matter-mineral complexes, it is possible that direct toxicity will be more prominent in the case of relatively hydrophobic TiO_2 NPs [55]. In addition, Ge et al. recently reported that TiO_2 NPs in soil may change bacterial community composition and reduce bacteria diversity [56].

This study also demonstrated that SEM could be a powerful tool for assessment of morphological changes of cotton fabrics induced by attack of microorganisms in soil during the biodegradation process [49]. Figure 4 shows the appearance of the control cotton fabric before testing (a–c) and control cotton fabric (d–f), cotton fabric impregnated with Ag NPs from 50 ppm colloid (g–i), and cotton fabric impregnated with TiO_2 NPs from 0.1 M dispersion (j–l) fabrics after 3 months of biodegradation [49]. It is

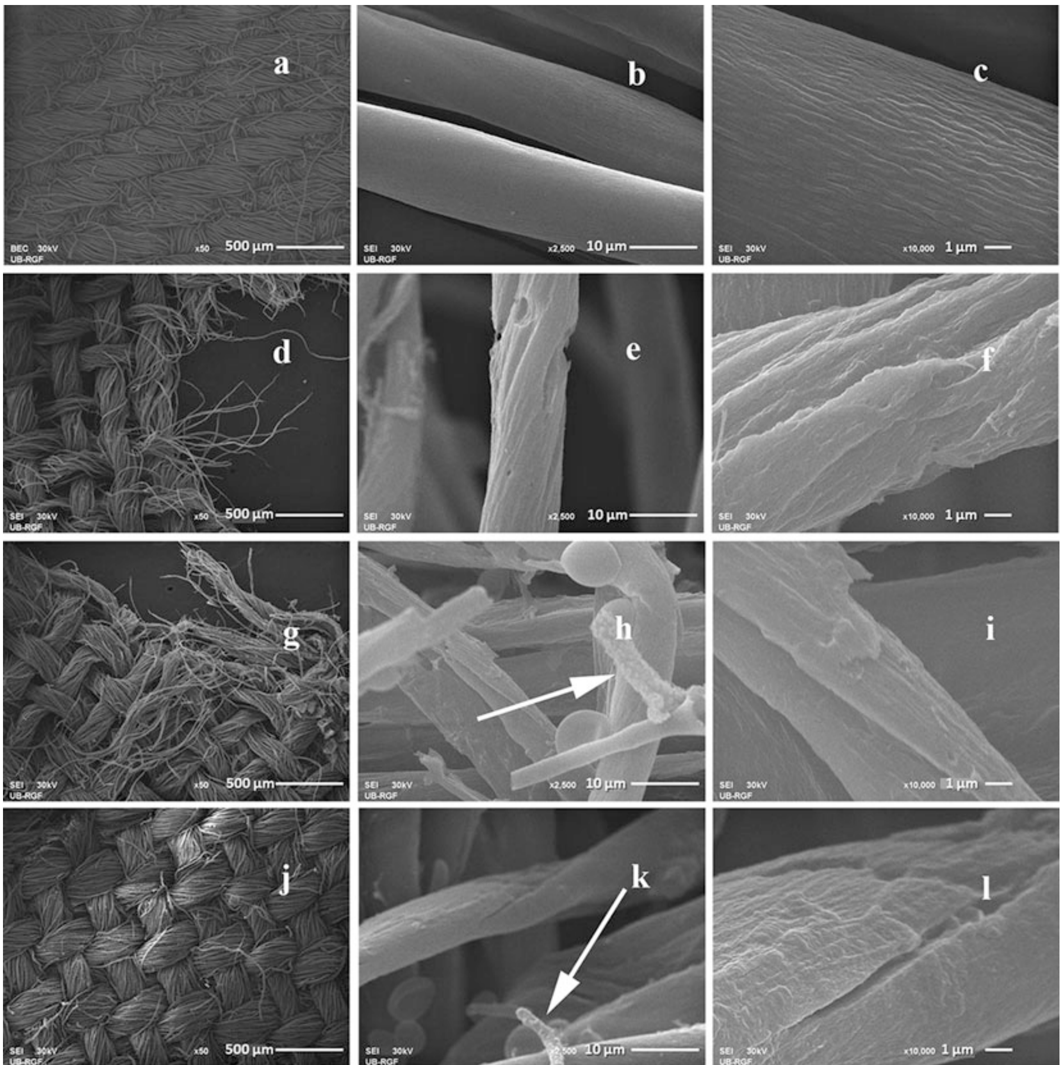

Fig. 4 SEM images of control cotton fabric before testing (**a**–**c**), control cotton fabric after 3 months of biodegradation testing (**d**–**f**), cotton fabric impregnated with Ag NPs from 50 ppm colloid after 3 months of biodegradation testing (**g**–**i**) and cotton fabric impregnated with TiO_2 NPs from 0.1 M dispersion after 3 months of biodegradation testing (**j**–**l**). Reproduced from [49]

obvious that severe damage of cotton fibers was more prominent on control sample and sample impregnated with Ag NPs.

The existence of fungus hyphae protruding from the fibers is evident in Fig. 4h and k (marked with arrows). Namely, Szostak-Kotowa indicated that cuticle layer of cotton fiber is subjected to digestion process first [57]. After cuticle layer elimination, fungi penetrate throughout the second wall until they get the cotton fiber lumen where they further grow. In fact, mycelium grows from the fiber interior toward the fiber wall secreting the enzymes that cause the degradation. Further, mycelium begins to sporulate on the

outer surface of the cotton fiber. In contrast, bacteria act in the opposite direction [57]. Tomšič et al. reported that fungi play a crucial role in biodegradation of cellulose fibers since bacteria need ambient with larger moisture content, i.e., cotton fabric should be saturated during the complete degradation process [45].

Milošević et al. analyzed the biodegradation behavior of cotton fabric and cotton/PET blend impregnated with Ag/TiO_2 NPs [50]. Ag NPs were fabricated by in situ photoreduction of Ag^+-ions on the surface of TiO_2 NPs deposited on the textile substrates [58]. The samples excavated from soil during the soil burial test were characterized in detail and their behavior during biodegradation was photographed in certain time intervals as shown in Fig. 5 [50]. Obtained results indicated that control cotton and cotton/PET samples lost nearly 50% of their weight in the first 25 days of testing. In contrast, cotton fabric impregnated with Ag/TiO_2 NPs remained practically intact in this period, but it underwent damage in the following two weeks to such an extent that weight loss could not be measured. On the other hand, the biodegradation of blend was much faster due to almost two times lower content of Ag compared to cotton fabric. However, both control blend and blend impregnated with Ag/TiO_2 NPs lost approximately 67% of their weight after 46 days of testing. In other words, the whole amount of cotton biodegraded in the samples as the blend consisted of 67 and 33 wt.% of cotton and PET, respectively. The images of both the samples after 46 days of testing made an impression that the fabrics were intact (Fig. 5), but they were actually more porous and when exposed to light it became evident that only yarns made of PET fibers remained.

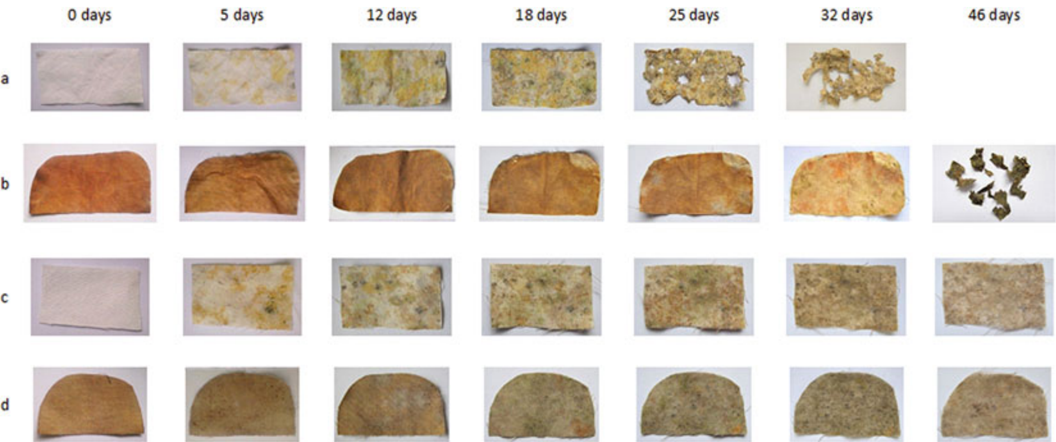

Fig. 5 The photographs of fabrics disintegration after certain time intervals of soil burial testing: (**a**) cotton fabric, (**b**) cotton fabric impregnated with Ag/TiO_2 nanoparticles, (**c**) cotton/PET fabric, and (**d**) cotton/PET fabric impregnated with Ag/TiO_2 NPs. Reproduced from [50]

The biodegradation of cotton in the blend was confirmed by the FTIR analysis. Namely, after 1 month of soil burial testing the bands in the FTIR spectrum of blend originating from PET became clearly noticeable. The damage of cotton fibers was also visible in the SEM images of the blends [50].

Obtained results indicated that Ag/TiO_2 NPs inhibited biodegradation of cotton and cotton/PET blend but less flagrant than in the previous study where the influence of Ag and TiO_2 NPs on biodegradation of cotton was studied separately [49]. Less pronounced inhibition of biodegradation of samples in the latest study is suggested to be due to different composition and structure of nanocrystals, higher temperature (29 °C vs. 18 °C), and soil water content (96.5% vs. 54%) and type of soil. The type of soil plays an important role in the biodegradation process as it may contain various enzymes and microorganisms. Park et al. indicated that microorganisms in soil are generally more active at higher temperature and humidity [4].

Recently, Primc et al. made a contribution to this research field by investigating the biodegradability of cotton fabrics activated by oxygen plasma and impregnated with ZnO NPs [51]. Macroscopic changes in the fabrics were visually detected already after 6 days of soil burial, but distinction between biodegradation behaviors of samples was more prominent after 12 days. Untreated and plasma treated samples were completely disintegrated into pieces after 12 days of soil burial which indicated their high biodegradability. In contrast, the only sign of biodegradation in the samples impregnated with ZnO NPs was gray-brownish staining that was explained by higher resistance against biodegradation. Visual observations were in good correlation with SEM images of cotton fibers. The strongest fiber damage was evident on oxygen treated fabric and the weakest on the samples impregnated with ZnO NPs where only superficial cracks appeared. FTIR spectra of unburied and buried cotton fabric impregnated with ZnO NPs were similar. The only relevant difference was higher intensity of absorption band at 1640 cm^{-1} in the buried sample which correspond to amide I, i.e., proteins that were formed during microorganism's growth and that were irreversibly bound to the fibers. Obviously, in this sample competition between antimicrobial activity of ZnO NPs that suppressed the biodegradation process and effects of plasma treatment which facilitated biodegradation took place. The results undoubtedly pointed out that ZnO NPs hindered the biodegradation, but the mechanism of their action was not discussed. This mechanism should be elucidated in more detail since the ZnO NPs like previously discussed TiO_2 NPs [49] are semiconductors that demand UV light for their activation and these antimicrobial activity tests and reported experiments were carried out in the dark.

Plasma processing of textile materials was frequently applied for activation of textile surfaces prior to impregnation with metal and

metal oxide NPs in order to enhance the efficiency of their binding [20–25, 27–29]. This paper opens up new perspective of biodegradation examination: the influence of combined finishing/nano-finishing processes on biodegradation behavior of textiles has to be taken into evaluation.

5 Future Prospects

This chapter summarizes what is currently known regarding the effect of nano/finishing of textiles with metal and metal oxide nanoparticles on their biodegradation behavior. The review clearly shows that relevant data on this issue are necessary for acquiring essential knowledge required to reduce the doubts related to environmental exposure assessment of nanomaterials. Studies on biodegradation behavior were focused on the textile nanocomposites fabricated under laboratory conditions at this point. A number of textile products with incorporated nanoparticles are growing on the market. Thus, the research should be expended to commercial products. Undoubtedly, the research related to influence of nanoparticles characteristics on biodegradation should be continued. However, the complexity of the communication between nanoparticles/textiles and soil microorganisms poses new questions that should be answered by multidisciplinary research teams. Many efforts have to be made to develop the methods for routine monitoring of concentrations of metal and metal oxide nanoparticles in various environments (water, soil, sediments) as well as to discover their stability, reactivity, and mobility in these environments.

Acknowledgment

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Chapter 15

Evaluation of the Toxicity of Azo Dyes by *Allium cepa* and Study to Remove These Compounds in Aqueous Solution by *Saccharomyces cerevisiae*

Érica Janaina Rodrigues de Almeida, Guilherme Dilarri, and Carlos Renato Corso

Abstract

Textile industries are among the largest consumers of synthetic azo dyes. The effluents generated by them, especially during the dyeing stage of tissues, present high staining and concentration of organic loads. The disposal of azo dyes in the environment represents one of the main environmental problems, due to its high pollution potential. The recalcitrance from these compounds is due to the complexity of their molecular structures, which have a large number of aromatic rings, azo bonds, amines, amides, phenolic, and sulfonic groups. Their degradation is difficult and harmful to the organisms exposed to them. Several types of research aim to find treatments that are able to reduce the concentration of these compounds and, consequently, the metabolites that are generated from their degradation. Those treatments can often become more toxic than the initial molecule. Among these treatments, biological treatment has a prominent place because they present good efficiency and lower costs for its implantation. Thus, this paper aimed to study the toxicity of the synthetic azo dyes Acid Orange 7 and Direct Violet 51 using as test organism bulbs of *Allium cepa*. The toxicity of these compounds was evaluated before and after degradation treatment with *Saccharomyces cerevisiae*. One of the main concerns of the study was to use simple methods of analysis, so bulbs of *A. cepa* were chosen to evaluate the toxicity, and yeast *S. cerevisiae* to carry out the process of decolorization.

Key words Acid Orange 7, Direct violet 51, *Allium cepa*, *Saccharomyces cerevisiae*

1 Introduction

Dyes are organic compounds that have a conjugate system of single and double bonds. Generally, they absorb light in the visible region between 400 and 700 nm of the light spectrum. And they may have one or more chromophore groups. To control stability and solubility these molecules also have hydroxyl groups, sulfonic and carboxylic acids, as well as amine groups [1].

They are essential in the textile industry, but the main challenge that this industry encounters is the uncontrolled use of these

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compounds and the inefficiency of the dyeing processes. Between 10 and 15% of dye used in the dyeing processes are discarded as effluent [1]. Many are toxic, carcinogenic, and mutagenic, which makes them highly harmful to aquatic environments and organisms exposed to them.

It is estimated that about 100,000 different types of synthetic dyes and pigments are produced commercially. The most important group is the azo dyes, characterized by the presence of one or more azo bonds (R_1–N=N–R_2), corresponding 60–70% of the total synthetic dyes produced worldwide. Azo dyes are considered an important class of environmental contaminants, resistant to many types of treatments, difficult to be mineralized and persistent in the environment [2, 3].

Many methods have been employed in the treatment of azo dyes, including physic-chemical such as adsorption [3], oxidative such as ozonation [4], and biological treatments [2, 5]. However, each technique has its disadvantage, including operational costs and production of large quantities of sludge. In addition, most treatments are unable to remove high concentrations of azo dyes within a short time.

When compared, biodegradation methods are more reliable and efficient in terms of cost compared to physical-chemical methods. However, azo compounds and their metabolites are toxic to different microorganisms, which hinder the degradation process and the application of the technique efficiently, often preventing the success of the treatment.

Thus, the aim of the present study was to analyze the degradation of the synthetic azo dyes Acid Orange 7 and Direct Violet 51 by the yeast *S. cerevisiae* and verify the toxicity of these dyes before and after this treatment using as bioassays with bulbs of *A. cepa*.

2 Materials and Methods

2.1 Azo Dyes

Azo dye Acid Orange 7 (C.I. 15510) was obtained from Aldrich Chemical Company, Inc., the chemical used was FW 350.33, water-soluble, λ_{max} = 483 nm. Dye purity of 90% (Fig. 1).

And the azo dye Direct Violet 51 (C.I. 27,905) was also obtained from Aldrich Chemical Company, Inc., the chemical

O
S
O
O^-Na^+
N
N
OH

Fig. 1 The molecular structure of Acid Orange 7

Fig. 2 The molecular structure of Direct Violet 51

used was FW 719.71, water-soluble, $\lambda_{max} = 544$ nm. Dye purity of 50% (Fig. 2).

2.2 Microorganism

Biodegradation tests were performed with *S. cerevisiae*, obtained from biological yeast Fleishman®. To prepare the suspension, 30 g of biological yeast were dissolved in 100 mL of deionized water.

2.3 Toxicity Tests with A. cepa *Bulb*

To perform the toxicity tests, bulbs with a mean diameter of 1.50 and 2.00 cm were selected. Bulbs with these sizes allow better accommodation in test tubes during bioassays [6].

Before the start of each test, the bulbs were cleaned, the outermost dry leaves and tissue remnants and dried roots were removed, taking care to keep the ring of root primers intact.

In a normal population the existence of low developmental bulbs is expected, so before the beginning of each toxicity test, the bulbs were kept in pure mineral water for a period of 24 h or 48 h. In this first step, they were packaged in a well-drilled styrofoam sheet (to allow root ring contact with water) and then placed inside a tray of mineral water. After this period, only the bulbs that had already started the development of their roots were selected for the toxicity test. For each concentration of dye solutions, five bulbs that presented the same pattern of root growth were separated.

The phytotoxicity test was used to determine the inhibition of root growth of *A. cepa* bulbs before and after biodegradation treatments. For such, 10 mL of the test solution were added in each tube test and one *A. cepa* bulb. After this step, the tubes were placed in a climatic chamber at 21 ± 1 °C in the absence of light for 72 h. The positive control was $ZnSO_4$ 0.05 N and the negative control was mineral water. At the end of the exposure period, measurements of the roots were taken and growth inhibition was calculated using Eq. 1.

$$\text{In}\ (\%) = \frac{(Rgc - Rgs) \times 100}{Rgc} \quad (1)$$

in which In (%) is the percentage of inhibition, *Rgc* is the root growth negative control, and *Rgs* is the root growth in the dye solution.

2.4 Biodegradation

The biodegradation test with Acid Orange 7 and Direct Violet 51 dyes were carried out at the same concentration as the 50% inhibition of *A. cepa* root growth prior to decolorization treatment. The bioassays were conducted in test tubes containing 10 mL of the dye solution, which was autoclaved for 15 min at 120 °C. After the solution sterilization process, 4 mL of 30% *S. cerevisiae* yeast suspension was added, then the tubes were shaken and placed in an incubator at 30 ± 1 °C. The solutions were analyzed by UV-Vis spectrophotometry (Shimadzu UV-Vis-2401 PC) every 24 h. The solutions were analyzed in a quartz cuvette 5 mm in width.

Changes in the absorption spectra of each dye before and during treatment allowed us to evaluate the degradation of the molecules from the ratio absorbance values ($Abs^{\lambda max1}/Abs^{\lambda max2}$). Assuming that significant changes in these values indicate that the molecules are undergoing changes in its chemical structures, i.e., the biodegradation process is predominant. In contrast if the values remain constant, what is predominant in the system is the biosorption/adsorption process [7].

2.5 FT-IR Spectroscopy Analysis

Fourier transform infrared (FT-IR) spectroscopy analysis (Shimadzu FTIR-8300) was also performed to obtain more detailed information on the transformation of the dye after the biodegradation treatment. FT-IR spectra provide information on molecular structure and this method is a useful tool in the analysis of metabolites formed after the biotransformation of dye molecules. For the FT-IR analysis, samples were dried at 105 °C. KBr discs were then prepared at a ratio of 1 mg of sample: 149 mg of KBr [2]. The discs were placed in suitable holders and readings were performed in the mid-infrared region (400–4000 cm^{-1}) with 16 scans at a resolution of 4 cm^{-1}.

3 Results and Discussion

3.1 Toxicity Tests Before Biodegradation Treatments

The toxicity results for the Acid Orange 7 dye before the decolorization treatment determined that for the *A. cepa* bulbs, a low degree of toxicity was present, with an inhibition concentration of 50% (IC_{50}) equal to 3500 μg mL^{-1} (Fig. 3).

However for the Direct Violet 51 dye, in relation to the results obtained for Acid Orange 7, it was more toxic, with an IC_{50} value equal to 400 μg mL^{-1} (Fig. 4).

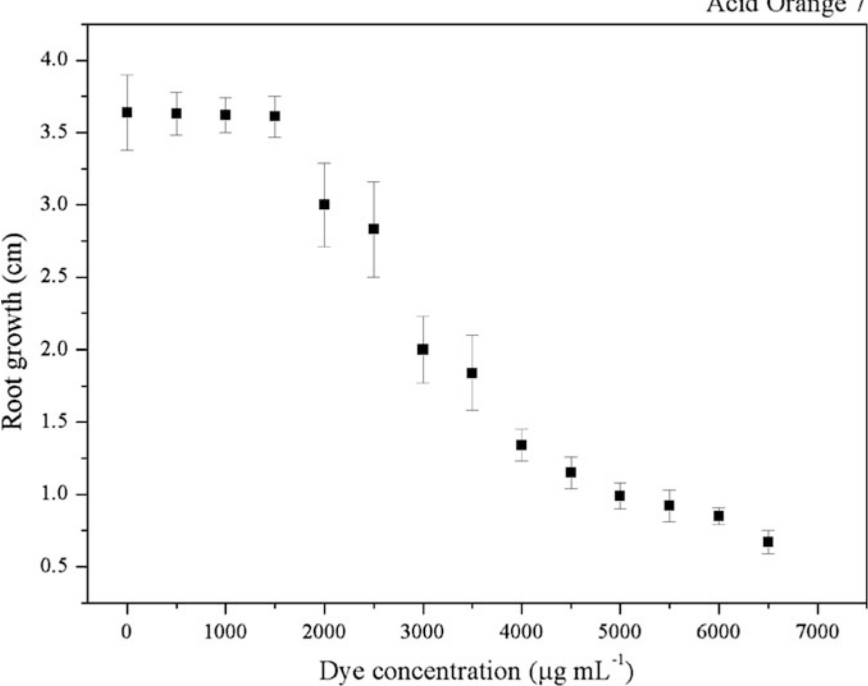

Fig. 3 Inhibition of root growth of *A. cepa* exposed to different concentrations of dye Acid Orange 7. [Root growth (cm) = −2,12,514−(0.0005271*dye concentration)] R^2=0.9390

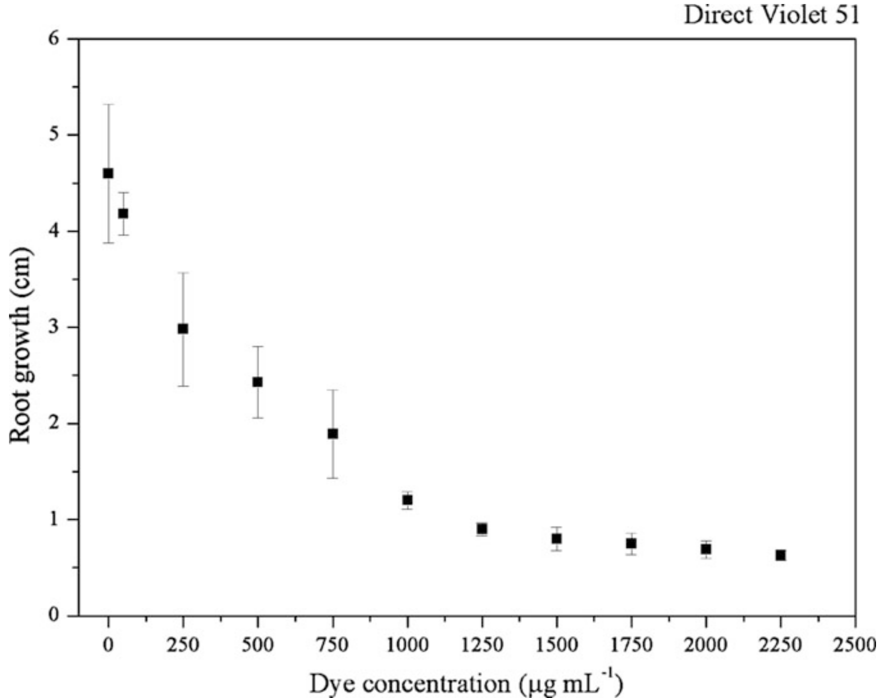

Fig. 4 Inhibition of root growth of *A. cepa* exposed to different concentrations of dye Direct Violet 51. [Root growth (cm) = −3,63,534−(0.00168*dye concentration)] R^2=0.8099

Thus, this study helped us to verify the concentration values of each dye that caused 50% inhibition of root growth. Therefore, it can be determined the initial concentrations of the solutions to achieve the biodegradation treatment with the yeast *S. cerevisiae*, being that our aim was to compare the toxicity changes before and after the bleaching process.

3.2 Obtaining of Dyes Spectra

Through the UV-Vis spectrophotometry analysis, it was possible to verify the maximum wavelengths of the experimental solutions of the dyes Acid Orange 7 and Direct Violet 51.

The first chromophore group of the Acid Orange 7 dye is in the visible region at 483 nm, while the second chromophore group can be observed in the 320 nm regions, and corresponds to the presence of azo bonds. Direct Violet 51 showed the wavelengths in the 544 nm region of the visible and 350 nm in the ultraviolet region.

The remaining concentrations of the dyes after the decolorization treatment were determined from their respective calibration curves, which can be verified in Figs. 5 and 6 respectively for Acid Orange 7 and Direct Violet 51.

3.3 Biodegradation Treatments

The UV-Vis absorbance spectra of the solutions treated with *S. cerevisiae* at different intervals of time showed changes in wavelength peaks, 483 and 320 nm for Acid Orange dye 7 (Figs. 7 and 8), 544 and 350 nm for Direct Violet 51 dye (Figs. 9 and 10). In Figs. 8 and 10 it is possible to verify the decolorization

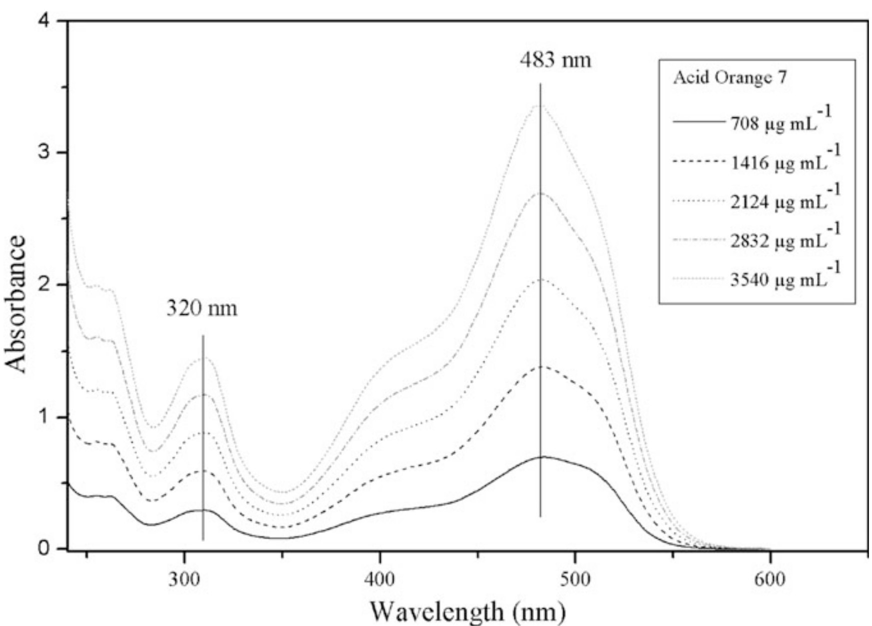

Fig. 5 UV-Vis spectrum of dye Acid Orange 7 at concentrations of 708, 1416, 2124, 2832, and 3540 μg mL^{-1}. Abs^{483nm} = 0.0347 + (0.0009*dye concentration). R = 0.999

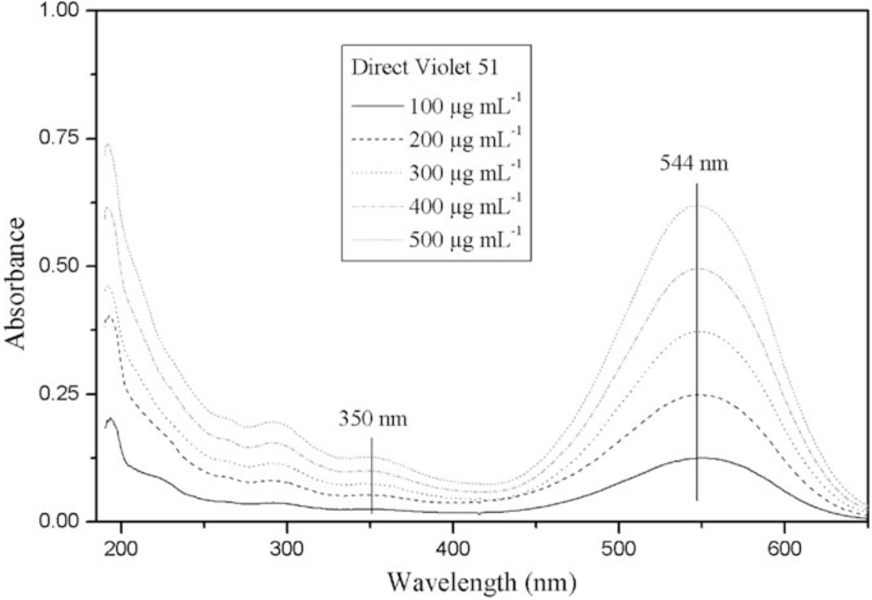

Fig. 6 UV-Vis spectrum of dye Direct Violet 51 at concentrations of 100, 200, 300, 400, and 500 μg mL^{-1}. $Abs^{544nm} = 0.0347 + (0.0009*\text{dye concentration})$. R = 0.999

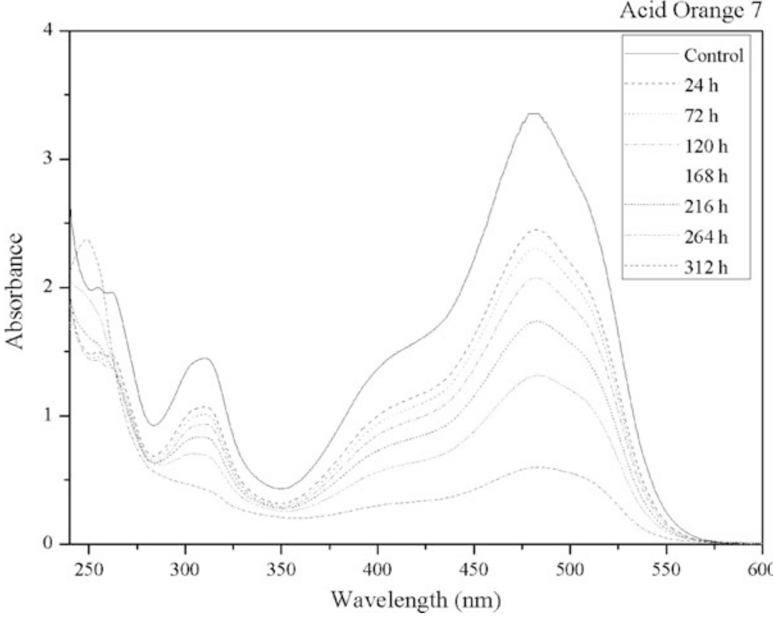

Fig. 7 UV-Vis spectrum of dye Acid Orange 7 after 312 h of treatment with the yeast *S. cerevisiae*

rates of the solutions after the treatment with the yeast and the variation of the ratio absorbance values.

Analyzing these results it is possible to affirm that the decolorization rate of the solutions increased proportionally to the time of

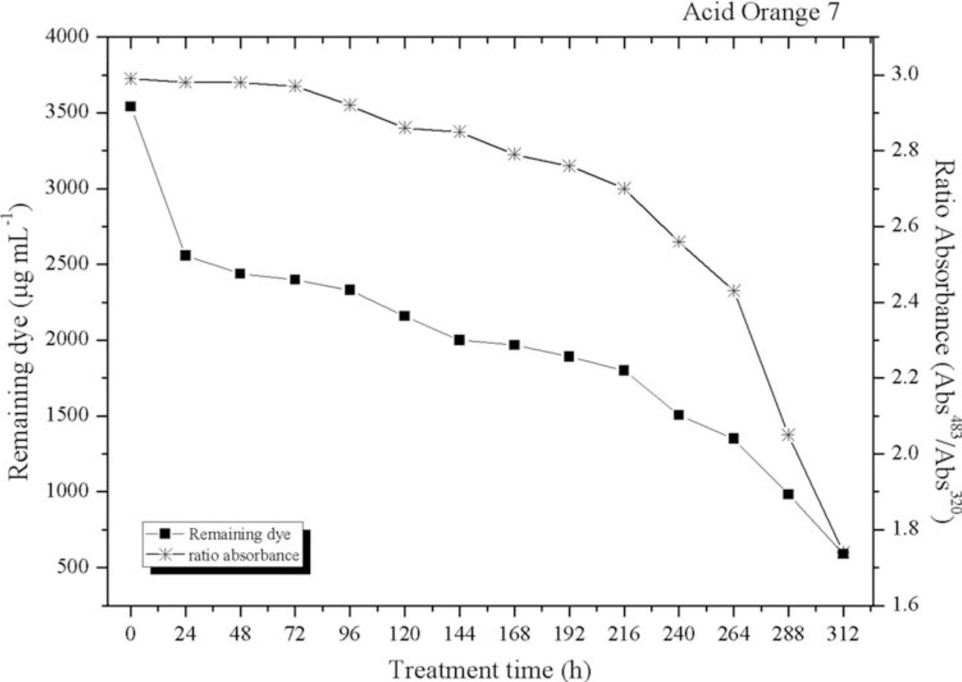

Fig. 8 Remaining dye and ratio absorbance of dye Acid Orange 7 after 312 h of treatment with the *S. cerevisiae*

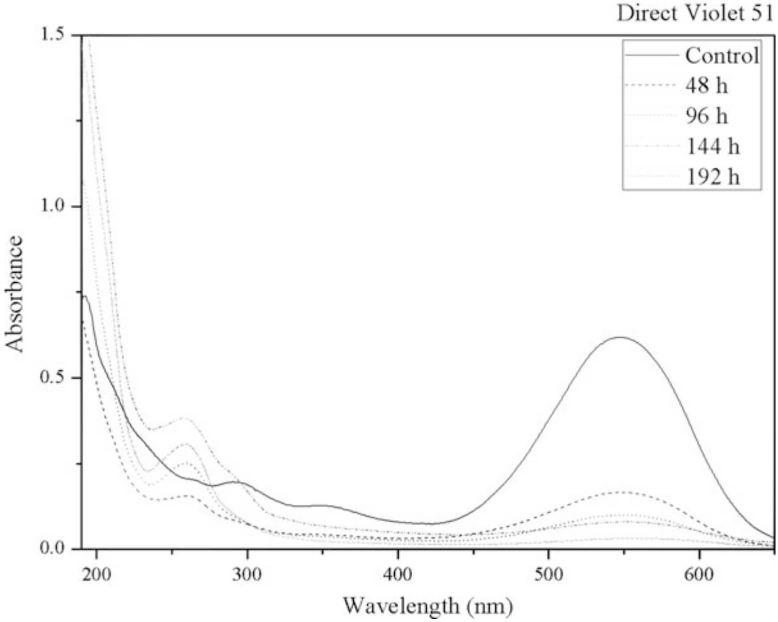

Fig. 9 UV-Vis spectrum of dye Direct Violet 51 after 192 h of treatment with the yeast *S. cerevisiae*

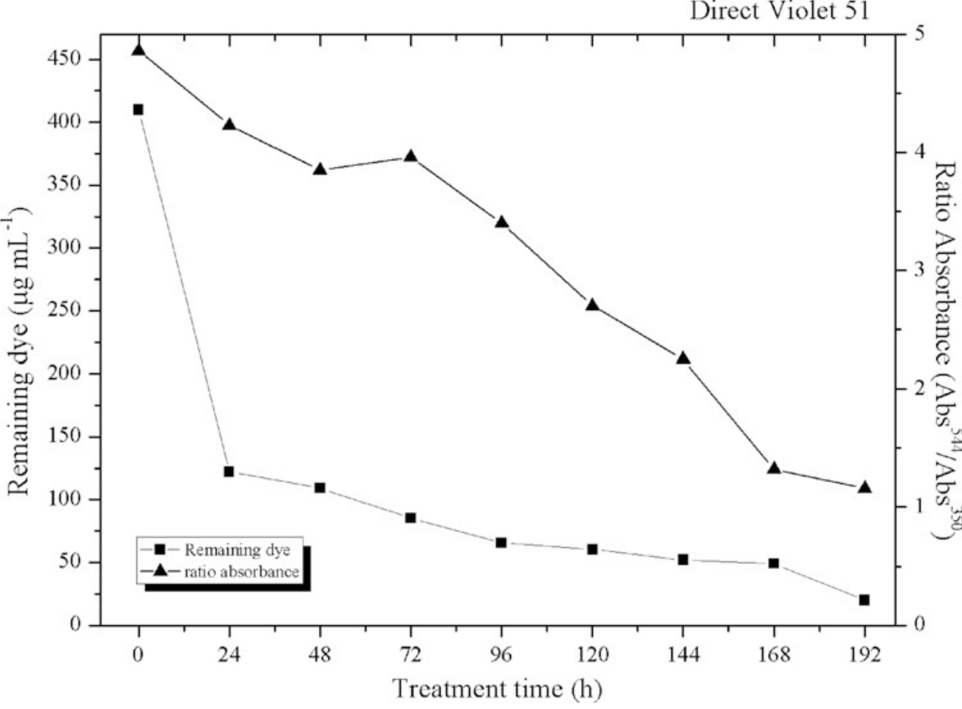

Fig. 10 Remaining dye and ratio absorbance of dye Direct Violet 51 after 192 h of treatment with the *S. cerevisiae*

contact with the yeast *S. cerevisiae*. Direct Violet 51 dye was degraded more rapidly, exhibiting complete decolorization in 192 h of the treatment, while Acid Orange 7 dye took 312 h to be completely discolored.

The ratio absorbance data indicate that for the Acid Orange 7 dye in the first 72 h of the treatment, the predominant removal process was adsorption/biosorption since there were no significant alterations of these values. Only after 72 h of contact with the yeast, the molecules began to be degraded, which is evident in the spectral changes of the UV-Vis spectrophotometry analysis, and consequently led to the alteration of the ratio absorbance values.

However for Direct Violet 51 dye, within 24 h of the treatment, the analyses indicated significant changes in their absorbance spectrum and consequently changes in absorbance ratio values, evidence for the higher affinity of the microorganism for the molecules of this dye.

The cell wall of *S. cerevisiae* consists mainly of polysaccharides, proteins, lipids, and has many functional groups that function as active sites for dye binding. Dye molecules can interact with these active sites on the cell surface through physical bonds such as van der Waals, or chemical as ionic or covalent interactions [8] when we speak of the removal of these molecules from the aqueous medium

by biosorption/adsorption processes. While the degradation process occurs mainly by the action of enzymatic complexes produced by the yeast, they are able to break the bonds of these molecules.

The analysis of FT-IR spectroscopy has gained prominence as an analytical method for biodegradation processes of textile dyes.

Although the infrared spectrum is a characteristic of a molecule as a whole, certain groups of atoms give rise to bands that occur at more or less the same frequency regardless of the structure of the molecule. It is precisely the presence of these bands characteristic of groups that allow the identification, for example, of metabolites formed after the degradation of the dyes [9].

Thus, the FT-IR spectra allowed us to observe the molecular structure of dyes in greater detail. When we compare the absorption spectra of the solutions before and after the treatment with the yeast, it is possible to see the structural changes that occurred in the molecules of the dyes.

In Figs. 11 and 12 we can observe the FT-IR spectra of the dyes Acid Orange 7 and Direct Violet 51 before and after the treatments of decolorization with the yeast *S. cerevisiae*.

Observing the FT-IR spectra of the solution of the Acid Orange 7 dye before and after the yeast treatment, it is possible to identify significant alterations in the 1228 cm^{-1} region, characteristic of sulfonic groups [10]. The reduction or disappearance of this peak indicates a breakdown of the bonds involving this functional group after the treatment. The 1381 cm^{-1} peak present in the

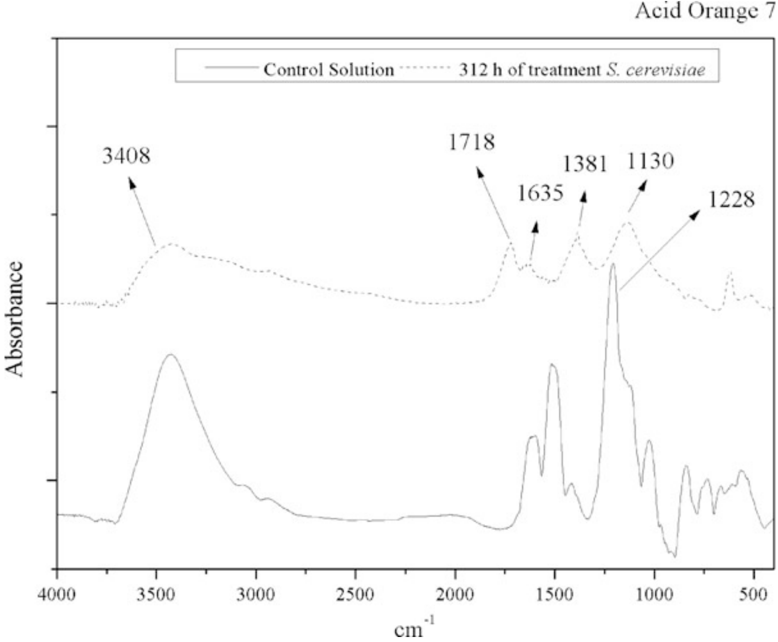

Fig. 11 FT-IR spectra of Acid Orange 7 before and after 312 h of treatment with *S. cerevisiae*

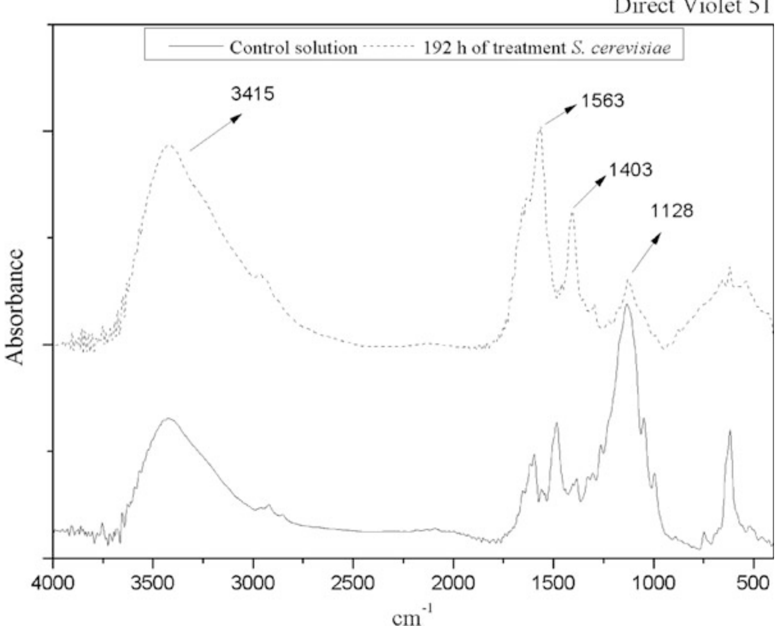

Fig. 12 FT-IR spectra of Direct Violet 51 before and after 192 h of treatment with *S. cerevisiae*

spectrum of the Acid Orange 7 dye after the treatment corresponds to the angular deformation of the –C–O bond [10]. The peak at region 1403 cm^{-1} in the Direct Violet 51 spectrum corresponds to the angular deformation of the –C–H bonds, and asymmetric deformation of the –C=C– bonds in aromatic rings [9, 11].

The presence of a broadband at 3408 e 3415 cm^{-1} in the FTIR spectra after the treatment for both dyes indicated the presence of water; however, this band also envelopes the OH^{-}stretching band of the carboxylic acid (RCOOH) moieties [12]. In the spectra of the Acid Orange 7, the presence of carboxylic acid in the product can be ascertained by the complimentary stretching band near 1718 cm^{-1} and 1635 cm^{-1} corresponding to the carbonyl (−C=O) group [10, 12].

After the treatment of both the dyes, there was a reduction in the spectral region of 1130–1128 cm^{-1}, a characteristic of –NH bonding vibration [13]. This reduction of intensity indicates the breakdown of these bonds in the initial molecules of the dyes, promoted by the action of azoreductase enzymes, generating amines as secondary metabolites. The spectral region of 1641 to 1403 cm^{-1} [14, 15] is also a characteristic of azo and amino groups, and the intense changes that occurred in this region reinforce the evidence of these metabolites in the middle of the chemical reaction.

Bioassays after the biodegradation process indicated a significant increase in the toxicity rate, and theoretically, the final concentration of dye remaining in each treated solution should not be

toxic, but instead, the inhibition of root growth was approximately 86% for both dyes studied. The rate of the root growth inhibition of solutions that were exposed to yeast was 50% (IC_{50}).

This increase in toxicity indicates that the dyes have undergone incomplete degradation and that the microorganism tested has not been able to degrade the metabolites formed. In addition, these intermediates were much more harmful to the test organism used in this study.

4 Conclusions

Analyzing the results we can conclude that the *S. cerevisiae* yeast was able to remove the Acid Orange 7 and Direct Violet 51 dyes from the aqueous solution. It was also able to degrade these molecules, and this process was confirmed during the UV-Vis and FT-IR spectrophotometry analyses, which showed intense changes in their spectra before and after contact with the yeast. The bulbs of *A. cepa* were sensitive to the different dyes concentrations tested, and the presence of metabolites with toxicity potential that was formed after biotransformation of dyes. But although the staining of the solutions was removed, in toxicological terms, the treatment proved to be inefficient as it was not able to degrade the metabolites formed after the breakdown of the dye molecules. Thus, bioremediation through biodegradation should be carried out for a longer period in order to achieve complete degradation of the intermediate compounds.

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Chapter 16

Ecotoxicological Characterization of Surfactants and Mixtures of Them

Francisco Ríos, Alejandro Fernández-Arteaga, Manuela Lechuga, and Mercedes Fernández-Serrano

Abstract

Surfactants are a wide group of chemical compounds, which have a large number of applications in agricultural, industrial, commercial, and household applications. Due to their extensive applicability, surfactants are usually found not only in wastewaters but also in natural waters at significant concentrations. Depending on the concentration in the aquatic environment, surfactants can be toxic for some living microorganisms. The adsorption of surfactants causes the depolarization of the cellular membranes and can cause acute and chronic effects on sensitive organisms through different nonspecific modes of action. Therefore, an ecotoxicological study of surfactants is needed to establish safe concentrations for the environment and compare with predicted or measured environmental concentrations. This chapter will gather the methods used to determine the ecotoxicity of surfactants as well as it will address the potential toxic effects of surfactants (including anionic, nonionic, and cationic) on representative organisms from different trophic levels. Furthermore, there is a growing interest in the study of the environmental properties of surfactant mixtures due to their co-occurrence in wastewaters and natural environments; therefore, this chapter will also address the synergistic/antagonistic effects on the toxicity of mixtures of anionic and nonionic surfactants. Finally, some challenges in the analytical procedures are fronted.

Key words Surfactants, Toxicity, Mixtures, Aquatic environment

1 Introduction

1.1 General Production Data

The detergents sector is a highly competitive and dynamic economic sector that is constantly innovating to meet the needs of consumers with increasingly safe and environmentally friendly products [1]. The most important market share in this sector belongs to important companies but there are also small and medium-sized companies that need new products and components to make new formulations.

One of the most important elements of cleaning and cosmetic products in general is surfactants, which constitute an important family of industrial chemicals and are widely used in almost every

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sector of modern industry. During the last decade global demand for surfactants has grown by around 300% and its current European production exceeds three million tonnes per year, most of which belong to nonionic surfactants (1.40 million tons) [2].

Global production of surfactants reached about 13 million tonnes per year in 2010, anticipated an annual growth of 2.8% by 2012 and between 3.5 and 4% thereafter [3]. In 2012, the world market for surfactants generated US $ 27.04 billion, the highest consumption area in Europe (31% of the total), followed by North America (28%) and China (17%). By 2018, it is expected to grow to $ 36,518 million with an annual growth rate of 6.19%, and the Asia-Pacific region to occupy the second position relegating North America to the third. Of this production, about 54% is used in detergents for textiles and household cleaning products, with only 32% for industrial uses.

New market trends require research of products offering not just high efficiency but also growing demand, meeting environmental legislation.

1.2 Applications

Surfactants are amphiphilic compounds (in their molecular structure they have a solvent and non-solvent group), but not all amphiphilic compounds can be considered surfactants, thus, ethyl alcohol is an amphiphilic compound, but it is not a surfactant. For an amphiphilic compound to be considered surfactant it is necessary that it has a hydrophobic chain length of eight or more carbon atoms (minimum hydrophobicity) and that it has a minimum polarity (suitable hydrophilic/hydrophobic ratio) depending on the characteristics of the group or polar groups present. On the other hand, these amphiphilic compounds must present the possibility of forming micellar aggregates to be considered surfactant compounds.

Molecular associations of surfactants determine phenomena related to their applications as:

- *Foaming*: the reduction of surface tension between a liquid and the air causes the liquid deformation and the inclusion of a multitude of air bubbles [4].
- *Emulsions, microemulsions, and liposomes formation*: when two immiscible liquids are present in the presence of surfactants, one of them, due to the drastic reduction of interfacial tension, can be divided, by mechanical action, into particles of small size (order of a few microns). This two-phase system divided into small droplets (dispersed phase) immersed in another phase (continuous phase) is called an emulsion. It is recognized by its milky or creamy appearance. It is thermodynamically unstable and eventually ends up separating into its two original phases (coalescence process). When the dispersed phase is formed by an apolar phase and the continuous phase is polar, the emulsion is

called oil-in-water emulsion (O/W), and conversely, when the dispersed phase is constituted by droplets of water or a polar substance, it is called water-in-oil emulsion (W/O). If the interfacial tension is very low, dispersed systems can be achieved in which the droplet size is less than one micron. In this case, the system is thermodynamically stable and is called microemulsion. Its appearance is translucent or totally transparent and of normally high viscosity. Emulsions and microemulsions are widely applied in cosmetics, pharmacy, food technology, etc. Liposomes are hollow complex structures, similar to a cellular structure, artificially formed by a system of water, oil, and surfactant (substances involved such as cholesterol, phosphatidylcholine, and lecithin). They are characterized by being able to transport active ingredients inside them, and above all by their ability to penetrate intact through biological membranes and later release, once the penetration has occurred, the active principle [5–7].

- *Formation of micelles*: in aqueous solutions the amphiphilic molecules form micelles in which the polar groups are on the surface and the apolar parts are immersed in the interior of the micelle in an arrangement that eliminates the unfavorable contacts between water and the hydrophobic zones, and allows the solvation of the groups of the polar chains. In other media, amphiphilic molecules can be organized as reverse micelles. When a low concentration of surfactant is present, the molecules are present as monomers. At higher concentrations, the free energy of the system can be reduced by aggregating molecules into micelles with the hydrophobic part located in the center and the hydrophilic head toward the solvent. The concentration at which this occurs is known as "Critical Micellar Concentration" (CMC). The CMC value depends on the type of molecule, namely its hydrophilic/hydrophobic ratio (and thus molecules with long hydrophobic regions have lower CMC values), ionic strength (nonionic surfactants have lower CMC than anionic and cationic surfactants [8]), and temperature. It is the formation of micelles in solution that gives the surfactant its properties of solubilization and detergency.
- *Solubilization*: for concentrations higher than CMC, the surfactant has the ability to solubilize hydrophobic compounds, resulting in completely solubilizing substances normally immiscible with each other. In transit, not only true solutions can be given but complex colloid or gel structures can be formed. In perfumery, this property is essential to make perfumes (oils) stabilize in a multitude of commercial products that must be perfumed (cosmetics, detergents, plastics, and other objects in general).
- *Detergency*: surfactants can cause that dirt particles cannot adhere to surfaces that "dirty," thanks to the modification of

the equilibrium of interfacial tensions of the system formed by the substrate, dirt, and washing bath (where the surfactant is dissolved). For this reason, surfactants are the main component of detergents.

- *Transfer of oxygen and other gases*: another of the most interesting effects of surfactants is the modification of the transfer of oxygen, and any gas in general, through membranes. Two are the systems that must be highlighted as very important: the first is the case of the lungs, since oxygen transfer is only possible because of the presence of so-called pulmonary surfactants. The other case is that of fish gills: when the aqueous medium in which they live is contaminated with slight amounts of surfactants, the fish end up dying. This is one of the reasons why it is imperative that surfactants are sufficiently biodegradable so that they do not reach rivers, lakes, and seas [9].

The above-mentioned properties give the surfactants a great versatility and the possibility of being used in many applications: pharmaceutical products, detergent formulations, metal operations, flotation, food, paints, etc.

The detergency is (at least in terms of tonnage) along with cosmetics, the main application of surfactants [10], these two fields account for more than 60% of the needs. Perfumery is related as an auxiliary branch of these and to a lesser extent, the food and drug market.

1.3 Legislation

The increasing consumption of surfactants as well as their possible environmental impact are the main reasons why different institutions have adopted standards for the control and use of these substances. The first legislation was established in Germany in 1961, where the biodegradability of anionic surfactants was required to be 80%. Directives were subsequently issued fundamentally on the biodegradability of surfactants used in detergent formulations:

- The European Union Directive No. 67/548/EEC (European Economic Community) with the respective amendment No. 7 allows the classification of the surfactants and the different mixtures formulated according to the results of toxicity for the different surfactants assayed [11].
- Directive (73/404/EEC), which provided that:

  "Member States shall prohibit the placing on the market and use of detergents where the average biodegradability of the surfactants they contain is less than 90% in each of the following categories: anionic, cationic, nonionic, and amphoteric. The use of surfactants having an average biodegradability index of at least 90% shall not adversely affect the health of humans or animals under normal conditions of use [12].

- Directive 73/405/EEC examines the procedures by which the biodegradability of anionic surfactants contained in detergents can be determined [13].

These Directives were brought up to date with Council Directives 82/242/EEC and 82/243/EEC [14, 15]. Finally, in 2004, the European Parliament established the "Regulation (EC) No 648/2004 of the European Parliament and of the Council of 31 March 2004 on detergents" [16] repealing Directives 73/404/EEC, 73 82/243/EEC, and 86/94/EEC and Commission Recommendation 89/542 on the labeling of detergents [17, 18], with the aim of harmonizing uniformly Member States and simplifying their future adaptations. This regulation aims at greater protection of the environment, including more restrictive legislation, since it includes all types of surfactants and imposes more stringent testing methods on final biodegradability than on initial biodegradability for detergents. It is also intended to protect consumers, who are more protected against perfuming substances and preservatives that are present in detergents and can cause allergies. Specific labeling is introduced to inform consumers about the presence of this type of substances in detergents. The Regulation makes the labeling requirements for these allergenic substances mandatory by incorporating them into the new legislation. The regulation includes regulations on labeling requiring manufacturers to have the list of all ingredients listed in decreasing order of concentration as well as the address of a website where the consumer can consult the complete list of ingredients. In addition, the Regulation adds a supplementary control method for surfactants with poor solubility in water (amendment of Annex III).

Briefly, this regulation establishes that:

- Primary biodegradability shall be considered satisfactory at a minimum level of 80% as measured in accordance with the test methods specified in Annex II.
- The determination of the anionic surfactants in the tests will be carried out with the analysis of the active substance to methylene blue (MBAS).
- The determination of the nonionic surfactants in the tests shall be carried out with the Method of the active substance to bismuth (BiAS).
- The determination of the cationic surfactants in the tests shall be carried out with the Analysis of the active substance to disulfine blue (DBAS).
- The determination of amphoteric surfactants in the tests shall be using the following procedures:

If there are no cationics: method used in the Federal Republic of Germany (1989) DIN 38409, Teil 20 and in other cases the Orange II method [19].

Detergent surfactants will be considered biodegradable if the level of biodegradability (mineralization) is at least 60% within 28 days, measured according to the test methods specified in Annex III.

In the European Union, the policy of prevention and control of chemicals is coordinated through the regulation "Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on the classification, labeling and packaging of substances and mixtures" [20], as subsequently amended by Regulation Commission Regulation (EC) No 790/2009 of 10 August 2009 [21]. This Regulation seeks to harmonize the requirements for the classification, labeling, and packaging of chemicals and their mixtures in accordance with the international system approved by the United Nations for the protection of the health and safety of the population and the environment, as well as to improve the free circulation of chemical substances and their mixtures.

1.4 Current Trends (Structure-Toxicity Relationships)

The toxicity of the surfactants is highly dependent on the length of the hydrocarbon and/or ethoxylated chain, as well as on the purity of the chemicals tested [22]. In general, the toxicity of surfactants for aquatic cells and animals increases with the ethoxylated chain length, although this tendency may decrease for chain lengths of 14 carbon atoms [9].

Kimerle [23] studied toxicity for LAS homologs and found that acute toxicity increases with increasing hydrocarbon chain length. In general, linear chain anionic surfactants tend to exhibit greater acute toxicity than branched chain ones, although this effect is compensated by greater biodegradability of the firsts [23]. For the specific case of LAS, toxicity decreases for isomers with the aromatic ring furthest from the end of the alkyl chain, i.e., 2-phenyls are more toxic than the central isomers [24].

The toxicity of polyethoxylated nonionic surfactants is increased by decreasing the length of the ethoxylated chain [25]. Comparing the toxicity of ethoxylated fatty alcohols with those of ethoxylated alkylphenols, the latter are less toxic [26], although their products resulting from degradation (non-ethoxylated nonylphenol or low ethoxylation) appear to be somewhat "recalcitrant" because their biodegradation is slower than that of the corresponding non-ethoxylated fatty alcohol homologues with 1 or 2 ethylene oxide units.

In general terms, it is possible to affirm that there are a series of common criteria applicable to any type of surfactant:

1. The toxicity of surfactants in clean waters is greater than in contaminated waters, due to the adsorption of the surfactants on the suspended organic matter of the latter, thereby reducing the amount of surfactant in the solution.
2. The metabolites generated in the biodegradation process are less toxic than the starting products, with some exceptions, such as ethoxylated alkylphenols.
3. There is a relationship between biodegradation and toxicity, since the most toxic products are the most readily biodegradable.
4. The toxicity of surfactants is mainly due to the disruption they produce in cell membranes, which causes osmotic shock and alterations in the permeability of cells; which explains why most of the surfactants are more toxic to species of greater biological organization such as fish than to Daphnia magna.

2 Methodology in Ecotoxicological Characterization of Surfactants

Since ecotoxicity is one of the most important aspects to the assessment of the environmental impact of compounds, a large number of screening methods for aquatic toxicology evaluation have been developed. The use of toxicity tests is needed to establish concentrations safe for the environment and compare with predicted or measured environmental concentrations [27, 28].

Surfactant toxicity tests are generally conducted on organisms during a specific time period of the organism's life cycle, in which the toxicity is measured as growth inhibition, decrease in their reproductive capacity, immobilization, or mortality [29]. These tests comprise organisms from all the trophic levels, including bacteria, algae, crustaceans, and fishes and they have been standardized by important organizations such as the Organization for Economic Co-operation and Development (OECD), International Organization for Standardization (ISO), United States Environmental Protection Agency (EPA), or the European Commission (EC). The objective of ecotoxicity is not only to protect individual species, but rather the whole ecosystem. Although it is still under concern, it can be considered that if most of the organisms are protected, the whole ecosystem is preserved [30].

Toxicity assessment can be done through acute or chronic toxicity test. Acute toxicity describes the adverse effects of a substance that result either from a single exposure or from multiple exposures in a short period of time. In chronic toxicity tests, on the other hand, adverse effects are the result of long-term exposure to a

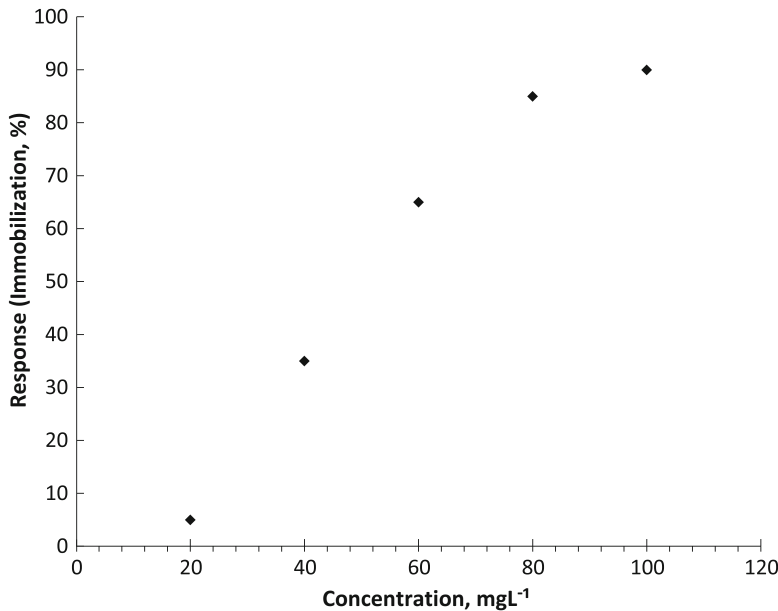

Fig. 1 Dose-response curves of a non-ionic surfactant to microcrustaceans *D. magna*

toxicant or other stressors that lasts during an entire life cycle [31]. The aim of the surfactant toxicity tests is the determination of the surfactant concentration which causes a certain percentage (X) of dead or affected organisms, in most cases 50%. This concentration is referred to as lethal concentration, (LCX) or effect concentration, (ECx). The test organisms are exposed to a range of surfactant concentrations and a dose-response curve is drawn. Fig. 1 shows an example of the dose-response curve of microcrustaceans *Daphnia magna* to a nonionic surfactant. From these graphs a mathematical relationship can be deduced between the effect and concentration, and the LCx or ECx is determined. This way, the lower the LCx or ECx are, the more toxic is the surfactant tested.

The variety of testing organisms in surfactant toxicity is wide (Table 1). Among them, microcrustaceans *Daphnia magna* is one of the most used to determine toxicity [27, 42], most prominent characteristics are its small size and short life cycle. Algae are sensitive to a large number of contaminants, such as insecticides, metals, industrial organic compounds, and surfactants and [43] reported that bioassays using *Pseudokirchneriella subcapitata* as test organism can be used to compare toxic effects of certain pure surfactants or their mixtures. In case of bacteria, various generous have been employed to toxicity assessment, such as *Pseudomonas putida* or *Photobacterium phosphoreum* [44–48]. Bacteria and particularly *P. phosphoreum* has been reported as the most sensitive assay, while testing a wide range of surfactants compared to other organisms and comparability between tests based on them is excellent [28, 49, 50]. In addition, for decades conventional aquatic fish

Table 1
The main test organisms to determine the ecotoxicity of surfactants in the aquatic environment

Organism test	Species	Effect	Duration	Reference
Bacteria	*V. fisheri* *P. putida*	Luminescent inhibition Growth inhibition	15 min 72 h	[32] [33]
Freshwater algae and cyanobacteria	*P. subcapitata* *D. subspicatus* *N. pelliculosa* *A. flos-aquae* *S. leopoliensis*	Growth inhibition	72 h	[34]
Marine algae	*S. costatum* *P. tricornutum*	Growth inhibition	72 h	[35]
Aquatic plants	*Lemna sp*	Growth inhibition	7 days	[36]
Microcrustacenas	*D. magna* *D. magna* *A. salina*	Immobilization Reduction in reproductive output Mortality	24–48 h 21 days 24 h	[37] [38] [39]
Gastropods	*P. acuta Drap*	Immobilization	24 h	[40]
Fishes	*Brachyctonio rerio (Sebra fish)* *Pimepjoles promelas* *Cyprinus carpio* *Orysias latipe* *Poecilia reticulata* *Lepromis macrochirus* *Oncorhynchus mykiss*	Mortality	96 h	[41]

bioassays were also routinely required for chemical. However, in many states, the use of fish species in toxicity test has given rise to both economic and ethical concerns [51] and some legislation bans or recommends replacing testing in fishes due to animal welfare concerns [52, 53].

3 Ecotoxicity of Surfactants

EcotoxicityThe activity of a chemical toward a living organism depends upon its physical and chemical properties, dependent on its structure. In the field of acute aquatic toxicity, many chemicals have been found to act by narcosis mechanisms, with toxicity being dependent on the ability of a compound to partition from the aqueous environment into lipid membranes. This process can be modeled by log P [54]. Nonionic surfactants behavior is well predicted by the Könemann's equation [55]. Anionic surfactants have been shown to act as polar narcotics. Cationic surfactants present the difficulty of applying these equations in the calculation of log, more complex because of the influence of cation N+ [54].

3.1 Anionic Surfactants

The class of anionic surfactants is particularly important, accounting for 60% of the world production [56]. Historically anionic surfactants are the oldest and the most common type of surfactants. Although linear-chain alkylbenzenesulfonate types are the most popularly used synthetic anionic surfactants, there has been a concentrated effort over the past few years to develop surfactants and builders with improved biodegradability and also non-polluting characteristics [57]. This growing concern has led to the development and use of other surfactants, such as the ether carboxylic derivative surfactants [28].

Liwarska-Bizukojc et al. [40] studied the toxicity of LAS, Alkyl sulfates, and Alkylpolyoxyethylene sulfates to three aquatic organisms: *Physa acuta Draparnaud*, *Artemia salina*, and *Raphidocelis subcapitata*. These authors investigated the relation between toxicity and molecular weight, founding that toxicity to all aquatic organisms studied increased with molecular weight of the surfactants. They also compared the toxicity of these anionic surfactants with some nonionic surfactants, confirming higher toxicity of nonionic surfactants in comparison to anionic ones.

Jurado et al. [44] studied the toxicity of three ether carboxylic derivatives surfactants and their binary mixtures to the bioluminescent bacteria *Vibrio fischeri*, the freshwater crustacean *Daphnia magna* and microalgae *Selenastrum capricornutum*. Results showed that according to the amendment N° 7 of the European Union Directive N° 67/548/ECC [11], the surfactant EC-R12-14E3 must be included in class II toxicity (R51), which means toxic against aquatic organisms; meanwhile, EC-R12-14E10 and EC-R8E8 are classified as harmful (class III R52) and safe, respectively. The results showed that *Vibrio fischeri* was more sensitive to toxic effects from ether carboxylic derivative surfactants than *Daphnia magna* and Microalgae were and that the toxicity is lower for the surfactant with the shortest alkyl chain. The degree of ethoxylation has the reverse effect: the higher degree of ethoxylation the lower toxicity.

Lechuga et al. [28] completed these results with the 72-h algal growth-inhibition test with the marine algal *Phaeodactylum tricornutum* and new ether carboxylic derivative surfactants with different chain length (R) and degree of ethoxylation (E), confirming the conclusion established by Jurado et al. [44]. These authors also present the structure-activity relationships for anionic surfactants as a function of log K_{ow} (octanol-water partition coefficient) (Table 2).

3.2 Nonionic Surfactants

Jurado et al. [59] studied the relationship between toxicity and metabolites during the process of biodegradation for fatty-alcohol ethoxylates, nonylphenol polyethoxylate, and alkylpolyglucosides. These authors also studied the relationship between the toxicity and the structure. Table 3 shows the relationships found for FAEs.

Table 2
Structure toxicity relationships for anionic surfactants

SARs	Ecotoxicological end point		References
$\log(1/LC_{50}) = 0.70 (\log K_{ow}) + 2.23$	*Daphnia*. Hard conditions, LC_{50} in mol/L		[58]
$\log(1/LC_{50}) = 0.64 (\log K_{ow}) + 2.44$	*Daphnia*. Soft conditions, LC_{50} in mol/L		[58]
$\log(1/LC_{50}) = 0.76 (\log K_{ow}) + 2.46$	*Gammarus*. Hard conditions, LC_{50} in mol/L		[58]
$\log(1/LC_{50}) = 0.71 (\log K_{ow}) + 2.27$	*Daphnia*. Soft conditions, LC_{50} in mol/L		[58]
$\log(EC_{50}) = -0.19 (R) + 0.11 (EO) + 2.59$	15 min *V. fisheri*, EC_{50} mg/L	EC	[28]
$\log(EC_{50}) = -0.17 (R) + 0.11 (EO) + 2.46$	24 h *D. magna*, EC_{50} mg/L	EC	[28]
$\log(EC_{50}) = -0.19 (R) + 0.05 (EO) + 3.24$	72 h *S. capricornutum*, EC_{50} mg/L	EC	[28]

Table 3
Structure toxicity relationships for non-ionic surfactants

SAR	Ecotoxicological end point	Surfactant family	References
$\log(EC_{50}) = 0.223 (HLB) - 2.06$	15 min *V. fisheri*, EC_{50} mg/L	FAE	[59]
$\log(EC_{50}) = -0.19 (R) + 0.15 (EO) + 1.88$	15 min *V. fisheri*, EC_{50} mg/L	FAE	[59]
$\log(EC_{50}) = -0.38 (R) + 0.30 (EO) + 4.23$	48 h *D. magna*, EC_{50} μmol/L	AE	[22]
$\log(1/EC_{50}) = 0.87 (\log K_{ow}) - 4.87$	48 h *D. magna*, EC_{50} μmol/L	AE	[60]

Fernández-Serrano et al. [61] studied the interaction between different types of surfactants that often are used as co-surfactants in detergent formulations to establish the best way to determine which surfactants will work better together. The study of anionic and nonionic surfactants is particularly interesting. In order to establish synergisms between the different surfactants, Toxicity Unit values (TU) were calculated as the ratio between the measured EC50 value and the average of EC50 values for individual surfactants. In this case, binary mixtures measurements indicated that the least toxic mixture was formed by the surfactant having lower individual toxicity. It was also demonstrated antagonism for the toxicity of some binary mixtures of ether carboxylic derivative and amine-oxide based surfactants, $TU < 1$.

Jurado et al. [45] studied the toxicity of nonionic surfactants alkylpolyglucosides to *Vibrio fischeri*, *Daphnia magna*, and

Selenastrum capricornutum. Their results are consistent with previous works about that *Vibrio fisheri* is the most sensitive of the three species tested, and *Selenastrum capricornutum* the least sensitive.

Lechuga et al. [28] completed these data with toxicity data of alcohol ethoxylates [22, 40, 60], Polyoxyethylene alkylphenyl ether [40], fatty alcohol ethoxylate [62], nonylphenol polyethoxylate [45, 62], alkylpolyglucosides [63], and amine oxide [64].

The SARs for anionic surfactants are summarized in Table 3.

3.3 Cationic Surfactants

Quaternary ammonium compounds (QACs) are molecules with at least one hydrophobic long alkyl chain attached to a positively charged nitrogen atom. Naecz-Jawecki et al. [65] studied the toxicity of 15 quaternary ammonium compounds (QACs) in a battery of four bioassays comprising the bacterium *Vibrio fischeri*, two ciliated protozoa *Spirostomum ambiguum* and *Tetrahymena thermophia*, and the anostracean crustacean *Artemia franciscana*. The toxicity of the test compounds was very high, with $EC(LC)_{50}$ values varying from 0.11 to 70 mmol/L. Microtox was the most sensitive bioassay, while the crustacean test was the least sensitive. In this sense, the behavior is similar to anionic and nonionic surfactants [28].

The high toxicity obtained for these cationic surfactants is logical if we have into account that one of the main attributes of QACs is their biocide activity. Disinfectants should have high inhibitory and bactericidal/fungicidal activity against a wide spectrum of pathogenic bacteria and fungi. They have also high activity against nontarget microorganisms.

Jing et al. [66] estimated the toxicity (in terms of EC_{50}) of QACs on two freshwater green algae *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* and then compared their differential sensitivities and built QSAR models for the prediction of the toxicity of various QACs. They calculated 16 molecular structure descriptors for each compound. These descriptors could be classified into three major groups: topological, geometric, and electronic-modynamic. Topological descriptors include Ea, C, D, T_{Indx}, A, K, T_{Con}, and J. Geometric descriptors included M_E, σ_{xx}, σ_{yy}, σ_{zz}, and CL. Electronic-modynamic descriptors included E, R, and log P [67]. The MLR analysis showed that the toxicity of QACs on *C. pyrenoidosa* and *S. quadricauda* could be described by two parameters T_{Con} and CL among the 16 descriptors. These authors also compared the sensitivity of both organisms to QACs, resulting in that *C. pyrenoidosa* was more sensitive to nine QACs and less sensitive to only three QACs according to the pEC_{50}. The influence of the different structural descriptor is also studied, concluding that the toxicity of the QACs decreased with the increase in the values of the two parameters CL and T_{Con} for the QACs.

Qiu et al. [68] used toxicity data and molecular structures from Jing et al. [66] to build accurate and reliable QSAR models. Since geometric and electronic descriptors depend on the 3D coordinates

Table 4
Structure toxicity relationships for cationic surfactants

QSAR model	Ecotoxicological end point	References
$pEC_{50} = 2.525 + 0.006a - 87.3237T_{con}$	*Scenedesmus quadricauda*, EC_{50} mg/L	[68]
$pEC_{50} = 2.647 - 192.963T_{con} - 0.080CL$	*Chlorella pyrenoidosa*, EC_{50} mg/L	[66]
$pEC_{50} = 1.907 - 77.543T_{con} - 0.0737CL$	*Scenedesmus quadricauda*, EC_{50} mg/L	[66]

of the atoms, comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) were used to build 3D structural descriptors, overcoming the limitations of the conventional 2D model. Several authors have established that molecular alignment is the most sensitive parameter in 3D-QSAR analysis [69, 70]. Qiu et al. [68] used the most toxic QAC as the template to align the other compounds from the series by common substructure alignment.

These authors conclude that the larger a value or hydrophobic group generally brings stronger toxicity, and the higher molecular charge changeability may also cause higher toxicity.

In Table 4 the QSAR model is presented.

4 Environmental Properties of Surfactant Mixtures

The use of surfactant mixtures may be for certain purposes, as in the following cases:

1. *When two properties are desired at the same time, two substances can be combined, each possessing one of the necessary properties.* There will then be a mixture with independent and cumulative effects. A laundry powder containing a detergent, a foaming agent, and a sequestrant can thus be formulated. The sequestrant subtracts the divalent ions, and therefore allows surfactants to play their role better. In such a case the effects are additive and independent of each other.
2. *In order to obtain properly an intermediate between that of the two components.* In general, it is the same property with different degrees of intensity. The case of the surfactant mixtures where hydrophobicity is calculated by the HLB rule is one of the most usual examples. It also applies to mixtures of oils and electrolytes. In any case, it all depends on how the properties are combined to get an average value.
3. *When desired to obtain a new property, different from that of the original substances.* It is possible the presence of a new substance, in the chemical or physical sense of the term. It is the

case of systems where the components have a strong interaction between them. The sense of the mixture does not obey the notion of average. On the contrary, it presents very strong deviations and is often spoken of synergism. This case can often be considered an extreme situation of the previous case.

There are many examples of mixtures of different surfactant classes in detergent and cleaning formulations. Since it is a well-established idea that cationic and anionic surfactants cannot be present in the same formulation, mixtures are generally between anionic/anionic, cationic/cationic, nonionic/nonionic, amphoteric/amphoteric, anionic/nonionic, cationic/nonionic, or amphoteric/nonionic. However, synergism increases with the degree of charge difference [71, 72] meaning that synergism between anionic/anionic or nonionic/nonionic is less than that between anionic/nonionic or cationic/nonionic, which, in turn, is less than that between cationic/anionic. Consequently, the higher synergism is obtained by mixing anionic and cationic surfactants, and therefore, a better understanding of that system may broaden the horizon for household formulations [73]. Commercial mixtures frequently incorporate nonionic surfactants because they do not produce ions in the aqueous solution making them compatible with other types of ionic surfactants.

After use of many commercial products surfactant mixtures are present in the aquatic environment, Therefore, there is a growing interest in the study of the environmental properties of surfactant mixtures [61, 74–76], since in some cases antagonistic or synergistic toxic effects have been shown for some mixtures. For example, Hisano and Oya [75] found lower toxicity in binary mixtures between sodium linear alkylbenzene sulfonate, sodium dodecyl sulfate, sodium oleate, and alcohol ethoxylates, and Fernández-Serrano et al. [61] demonstrated antagonism for the toxicity of binary mixtures of ether carboxylic derivative and amine-oxide-based surfactants. Ionic surfactants show synergism with nonionic surfactants, because nonionic head groups electrostatically shield the ionic head groups on the surfactant molecules at interfaces so that they can stay closer together with less effect from repulsion [77].

Critical Micellar Concentration (CMC) is clearly related to toxicity: the lower the CMC (the greater the surface activity) the more toxic, depending on the organism assayed in the toxicity test. In general, the most sensitive microorganism to CMC variation is the microalga *Selenastrum capricornutum*, and the least sensitive *Vibrio fischeri* [61]. CMC values can also be used in the case of surfactant mixtures to correlate toxicity with the structure of the surfactant.

5 Challenges in the Analytical Procedures

Very few studies that have considered the toxic effects of final detergent formulates or, more specifically, taken the effects on algae into account, showing the ability of these mixtures to interfere with cellular permeability, affect cell mobility, and act as inhibitor of some specific functions, such as photosynthesis or biosynthesis and biodegradation pathways [78–80].

The main constituents of those detergent formulates are surfactants. Commercially available nonionic surfactants (technical grade) usually contain impurities and have a broad molecular weight distribution owing to the degree of ethoxylation or the distribution of the fatty acid used in the synthetic procedure. This implies the use of several analytical procedures and technics both in the determination of several surface properties of the surfactants and their chemical structures.

The above-mentioned fact affects primarily the determination of the CMC, a key parameter in the characterization of surfactants, which is the reference in several further studies. There are several examples in the literature that deal with the CMC measurements by several technics and methodologies [81–84]; apart from the compilation by Mukerjee et al. [85].

Concerning the CMC it is widely accepted that the transition from monomeric to micellized surfactant does not take place at a sharply defined concentration but within a concentration interval around the CMC, Furthermore, in several situations, CMC has no strict meaning. This statement accounts strongly with commercial surfactants, in particular with some nonionic, like the commercial polyoxytheylene glycerol ester which differ both in the number of ethylene oxide (EO) groups and the composition of the alkyl part of the molecules. The alkyl part of this surfactant shows a variety in its structure and length depending on the manufacturer or supplier. Even more if we consider only a manufacturer we have detected changes in the composition in different batches. This fact makes more difficult the study of the progress of the biodegradation processes and the quantification of the toxic effects of the generated molecular species on living organisms. The analysis of all the individual reactions that take place during the biodegradation is a challenging task from an analytical point of view, due to the complex mechanisms of the metabolic process and the variety of the microorganism involved which leads to many several molecular species.

Coconut oil, e.g., which is used in the synthesis of LevenolTM, consists mainly of ester of lauric acid which comprises 52% of the fatty acid of this oil. Other fatty acids present in coconut oil are caprylic (9%), decanoic (10%), myristic (19%), palmitic (11%), oleic (8%), and others (around 5%).

Concerning this kind of surfactants, it is noteworthy several attempts in the literature focus on the analysis of similar molecules. At that respect, one method to follow the hydroxylated surfactant is the Fourier transform infrared spectroscopy (FTIR) Analysis [86, 87]. FTIR spectra offer some singular data to detect changes in the surfactant molecules during the biodegradation process. The ether linkage (C–O–C) is normally expected to appear at ~1100 cm^{-1}. The absorption bands in the 3400–3200 cm^{-1} region of the spectrum arise from the O–H stretching vibration of the polymeric structure of polyethylene oxide and free OH in these surfactants. The strong band at 2855–2849 cm^{-1} is attributed to the symmetric stretching of the methylene group of the attached chain. The strong band in the range from 1112 to 1098 cm^{-1} has been suggested to arise from the coupling of the ether group with the C–O stretching band of the surfactants under consideration.

The other possibility of identifying the biodegradation intermediates is the use of high performance liquid chromatography with mass spectrometric detection persisted [88]. However, in commercial surfactants it is not possible to carry out a quantitative determination because there is lack of analytical standards both of the several molecular species which constitute the surfactants, and the biodegradation intermediates that appear along the biodegradation pathways.

Finally, research on course approaches the above-mentioned determination by using the mass spectrometry technique Matrix-Assisted Laser Desorption/Ionization coupling to Time of Flight Mass Spectrometry (shortened to "MALDI-TOF-MS"). Common matrixes used are 5, 10, 15, 20-tetrakis(pentafluorophenyl) porphyrin and (2,5-dihydroxybenzoic acid). This methodology is considered appropriate to characterize some nonionic surfactant, mainly alkylpolyglucosides [89], and is useful to determine the average molecular weight. However, to the best of our knowledge, its application to study the degradation processes in the environment of these surfactants is a challenge, which is a key point to establish the toxic effect properly.

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Chapter 17

Whole Effluent Toxicity Assessment of Industrial Effluents

Takashi Kusui, Yasuyuki Itatsu, and Jun Jin

Abstract

Direct discharge of industrial effluents into aquatic ecosystems continues to be an important area of concern because of potential ecotoxic impact on biota in receiving water. Biological tests with aquatic organisms have the capacity to respond to chemicals and quantify their effects even if they are present as mixtures or are unidentified. To compensate for the shortcomings of traditional effluent regulation, whole-effluent toxicity (WET) testing was introduced in the USA, Canada, European countries, and South Korea. Additionally, to reduce the toxicity levels detected in effluents, a procedure called toxicity reduction evaluation (TRE) was proposed. Based on the lessons learnt in the implementation of WET testing in these countries, this approach, involving chronic assays, was suggested for introduction in Japan. Here, we present a short review on the bioassay-based regulations and biological methods prevalent in these countries. Furthermore, we introduce two case studies from Japan. The first study reports on short-chronic assays used as WET tests whereas the second discusses TRE and the application of toxicity identification evaluation (TIE) to identify causative factors, employing a combination of biological tests and physiochemical manipulations. We also discuss simple and rapid bioassays for routine monitoring of effluent toxicity.

Key words Whole-effluent toxicity (WET), Bioassay, Industrial effluent, Toxicity reduction evaluation (TRE), Toxicity identification evaluation (TIE)

1 Introduction

Direct discharge of industrial effluents into aquatic ecosystems continues to be an important area of concern because of the potential ecotoxic impact on receiving water biota. Due to the usually unknown, complex, and often highly variable composition of effluents, it is very difficult to predict and control their effects on aquatic ecosystems based only on measured chemical parameters in effluent standards. In order to compensate for the shortcomings of traditional effluent regulation, WET tests have been introduced in the USA, Canada, European countries, and South Korea [1–3]. The advantage of this approach includes aggregation of toxicity

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measured, detection of unknown toxicants, and measurement of bioavailable toxicants [4]. Aquatic bioassays have been used for effluent management as a standard designated to protect the receiving environment and reduce emissions to the environment [3].

The purpose of this chapter is to provide a short review on the bioassay-based regulations and biological methods prevalent in these countries. Then we introduce two case studies from Japan. The first study reports on short-chronic assays as WET tests whereas the second discusses TRE and the application of TIE to identify causative factors, employing a combination of biological tests and physiochemical manipulations. We also discuss simple and rapid bioassays for routine monitoring of effluent toxicity.

2 Brief History of Effluent Control Based on Biological Testing

In the early times of effluent bioassays, they were used, much like canaries were used by coalminers to alert for gaseous pollution, as indicators of water pollution. In the United states, first trial of effluent monitoring based on bioassays began in the 1940s; then the first attempt at standardizing effluent toxicity tests occurred in the1950s [5].

Under the Clean Water Act of 1977 established in USA, the narrative standard states that waters shall be free from “toxics in toxic amounts.” To ensure this standard, WET tests had been developed and standardized as part of the US National Pollutant Discharge Elimination System (NPDES) [1]. The series of methods, TIE and TRE, had been also developed to identify the cause substances and/or source and reduce the toxicity of effluents [6–8]. Application of these methods is discussed in Sect. 5.

In Canada, pulp and paper mill effluents were regulated with fish acute toxicity tests to ensure no acute lethality under the fishery law since 1975. In 1995, the regulation was revised to include a choice of either a fish or daphnia acute test.

In the EU, different approaches using effluent bioassays were employed in each member country. Since 2011, South Korea has introduced a daphnia immobilization test for effluent discharge limits as the first case in Asia and Oceania [9].

The present status concerning the regulatory use of effluent bioassays is summarized in Table 1.

As for the type of bioassays used, most common species are algae, invertebrate, and fish because they represent different eutrophic levels (primary producers, primary/secondary consumers) of natural ecosystems (Table 2). Freshwater and saltwater species are used depending on the type of receiving water environment, although freshwater bioassays are more widely applied.

Table 1
State of current use of effluent bioassays

Location	Regulatory use	Partial regulatory use	Research and development
Europe	Germany France Northern Ireland Sweden	Norway Spain United Kingdom	Belgium Denmark The Netherlands
North America	United States Canada	–	–
Asia and Oceania	South Korea	Australia New Zealand Hong Kong, SAR, China	Japan China

Most of the information was based on the review (2)

Table 2
Type of bioassays used for effluent management

	United States	Canada	South Korea	United Kingdom	Germany
Test sample	Effluent Receiving water	Effluent Receiving water	Effluent	Effluent Receiving water	Effluent Receiving water
Phyla used	Algae Invertebrate Fish	Algae Invertebrate Fish	Invertebrate	Algae Invertebrate Fish	Bacteria Algae Invertebrate Fish
Endpoints	Acute Chronic	Acute Chronic	Acute	Acute	Acute
Type of water	FW and SW	FW and SW	FW	FW and SW	FW

FW freshwater, *SW* salt water

3 Introduction of WET Approach into Japan

As shown in Table 1, effluent bioassays have not been used in a regulatory framework in Japan. However, the Japanese Environmental Ministry established an advisory body in 2009 to examine the feasibility of introducing the WET approach into the present regulatory framework. In March 2013, draft guidelines for WET testing were released, which proposed three freshwater short-term chronic tests (algae, crustacean, and fish). So far, there are few reports on WET testing of industrial wastewater in Japan [10] and there is an urgent need to expand knowledge of WET testing and better comprehend the ecotoxic potential of present industrial effluents before introducing the new approach.

4 Pilot Study of WET Testing for Industrial Effluents in Japan

In this section, we introduce the results of a pilot study according to the proposed WET tests [11, 12]. The test conditions used in this study are summarized in Table 3.

Final effluents were collected from industrial plants mainly located in Toyama Prefecture, Japan, from 2012 to 2016. For each bioassay, organisms were exposed to effluents at a series of dilutions (0, 5, 10, 20, 40, and 80%). Some samples with high toxicity were diluted to less than 5%.

The algal growth inhibition test was carried out with the green microalgae *Pseudokirchneriella subcapitata* (NIES-35) in accordance with OECD test guideline TG201 [13]. Algal suspensions (60 mL) inoculated at 0.5×10^4 cells/mL in an Erlenmeyer flask (200 mL capacity) were exposed to a range of effluent concentrations prepared with AAP or OECD medium. Samples were prepared in triplicate for each effluent concentration. The samples were incubated under continuous illumination from fluorescent lamps (ca. 60 μmol/m^2/s) at a temperature of 23 ± 2 °C in an orbital shaker. Algal cell density was determined with a particle counter (detection range 3–12 μm, CDA-500, Sysmex, Japan)

Table 3
Test conditions of short-term chronic bioassays

Test	Algal growth inhibition test	Cladoceran reproduction test	Short-term toxicity test on fish embryo and sac-fry stages
Test species	*Pseudokirchneriella subcapitata*	*Ceriodaphnia dubia*	*Danio rerio*
Organisms	Exponential phase of growth	neonates(<24 h old)	fertilized eggs(<4 h)
Duration	3 days	max.8 days	9 days
Test vessel	200-mL Erlenmeyer flask containing 60 mL of test solution	glass containing 15 mL of test solution	glass containing 50 mL of test solution
Replicates per treatment	Six replicates for control; three replicates for each test concentration	Ten replicates	Four replicates
Light	Continuous	16 h light: 8 h dark	16 h light: 8 h dark
Temperature	23 ± 2 °C	25 ± 2 °C	26 ± 1 °C
Test medium renewal	No	Every 2 days	Every 2 days
Endpoints	Growth rate (NOEC)	Reproduction (NOEC)	Survival and hatching (NOEC)

every 24 h in the 72-h growth test. EC_{50} (50% of effective concentration) and NOEC (no observed effect concentration) for growth rate were determined with analysis software (Ecotox-Statics ver. 2.6, The Japanese Society of Environmental Toxicology, Japan).

Cladoceran reproduction tests were assessed via a three-brood renewal toxicity test with *Ceriodaphnia dubia* standardized by Environment Canada [14]. At the beginning of the test, one neonate daphnia (<24 h old) was transferred to a glass containing 15 mL of diluted samples. Each treatment consisted of ten replicates of a particular test concentration or the control. During the test, the samples were incubated under illumination (light 16 h/dark 8 h) at a temperature of 25 ± 2 °C. Appropriate volumes of food (YCT and algae) were added daily and each test solution was renewed three times per week. The death of first-generation daphnia and the number of live neonates produced by first-generation daphnia were observed for 8 days. EC_{50} and NOEC for fecundity were determined with analysis software (Ecotox-Statics ver. 2.6). Figure 1 shows an example of results for this assay.

Short-term toxicity testing on fish sac-fry stages was conducted according to OECD TG212 [15]. Briefly, 10 fertilized eggs (<4 h) were placed in a glass containing 50 mL of test solution. Each treatment comprised four replicates of a particular test concentration or the control. During the test, embryos were incubated under illumination (light 16 h/dark 8 h) at a temperature of 26 ± 1 °C. Hatching and survival rates were observed daily during the test period (10 days). Based on survival and hatching rates, NOEC was calculated with analysis software (Excel Tokei ver. 6.0, Esumi Inc., Japan).

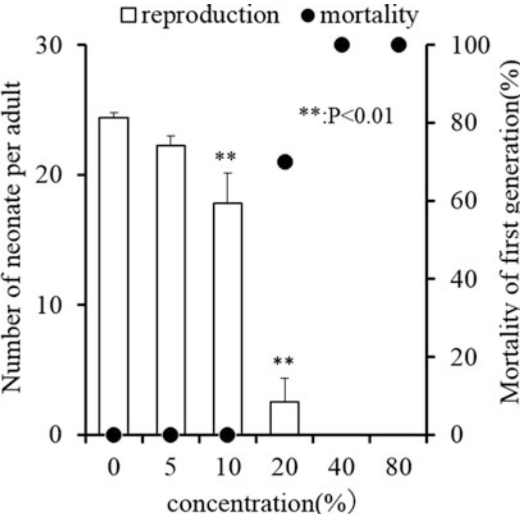

Fig. 1 Example of results for the cladoceran reproduction test. Data are presented as mean ± standard deviation ($n = 10$)

To compare the results of toxicity tests, chronic toxicity units (TUc) were calculated with the following formula:

$$TUc = 100/NOEC(\%)$$

In the case where NOEC equals 5% as shown in Fig. 1, the value of TUc is 20. The smaller the NOEC, which implies stronger toxicity, the larger the TUc becomes.

The results of WET testing for each industrial sector are summarized in Fig. 2. Among eight sectors, effluents from fabricated metal products plants as well as those from miscellaneous manufacturing industries showed relatively stronger chronic toxicity on cladocerans. On average, the order of sensitivity of the three organisms is cladoceran > algae > fish except for sewage treatment plant. This exception was explained by a preliminary experiment that monochloramine, which is a byproduct of disinfection, produces relatively higher toxicity to algae.

Overlapping profile of toxic responses is shown in Fig. 3a for three tests and in Fig. 3b for one or two tests. It is clear that chronic toxicity on fish occurred only when toxicity on the two other species appeared. As for cladocerans and algae, species-specific toxicity was observed as well as common toxicity. In terms of sensitivity, cladocerans showed the highest sensitivity, followed by algae as mentioned before (Fig. 4). The percentages of effluents with TUc ≧ 10 are 33, 55, and 3% for algae, cladocerans, and fish, respectively.

For a chronic criterion, USEPA proposed 1.0 TUc at the end of the mixing zone to prevent any further chronic toxicity in the receiving water [1]. In Japan, there are no environmental criteria

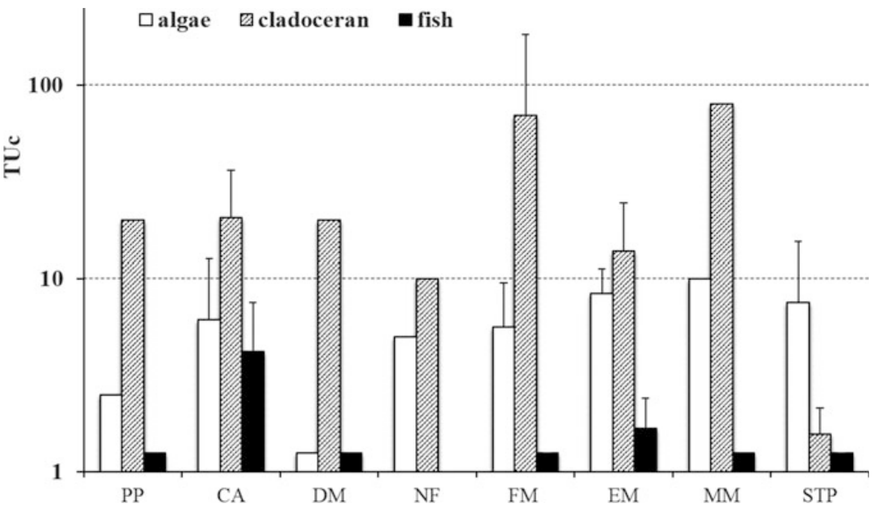

Fig. 2 Results of WET tests in each industrial sector; *PP* pulp, paper, and paper products (1); *CA* chemical and allied products (9), *DM* drugs and medicines (2); *NF* non-ferrous metals and products (1), *FM* fabricated metal products (8), *EM* electrical machinery, equipment and supplies (3), *MM* miscellaneous manufacturing industries (1), *STP* sewage treatment plant (9). Data are presented as mean ± standard deviation

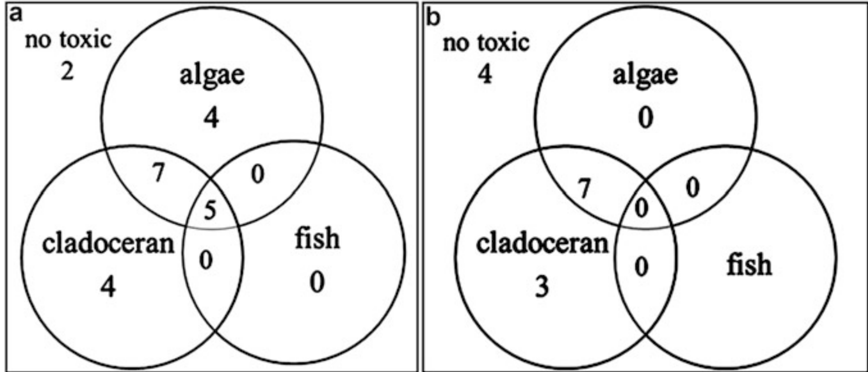

Fig. 3 (**a**) Overlapping profile of toxic responses: three tests ($n = 22$). (**b**) Overlapping profile of toxic responses: one or two tests ($n = 14$)

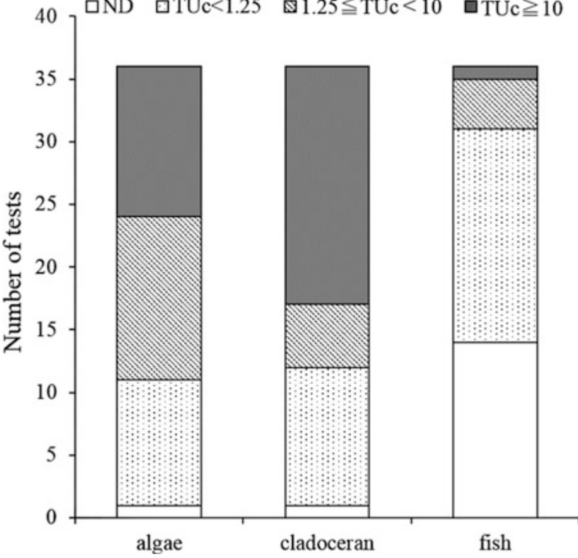

Fig. 4 Sensitivity of each tests ($n = 36$)

or standards based on units of toxicity; therefore, a TUc of ten was tentatively used as a criteria in this study. If the dilution factor was designated as ten, based on the rationale that most Japanese effluent standards are set to ten times the environmental standards of water bodies, a TUc of 10 was thus regarded as the chronic criterion. In this case, half of effluents exceed the criterion. In our region when a tenfold dilution was applied, it is likely to overestimate the environmental impact, but it rides on the safe side for environmental protection [11]. However, it is also clear that effects may be underestimated for some effluent. Thus, a further study is required to compare the WET measurements with impacts on receiving water and to provide a rationale for setting an appropriate dilution factor.

5 Case Study of Toxicity Reduction Evaluation

When the WET tests show unacceptable toxicity, a discharger is required to reduce the toxicity to meet criteria according to TRE. The purpose of TRE is to investigate a cause and to identify corrective actions for difficult effluent problems [1]. In this process, procedures for the characterization, identification, and confirmation of causative agents of effluent toxicity are designated as a Toxicity Identification Evaluation. Figure 5 shows the series of tests in Phase I TIE which characterize the toxic factor. By comparing the toxicity of samples treated chemically and/or physically with that of the original sample, the character of causative factors was estimated (Table 4).

The case studies of TIE were introduced below [12].

5.1 Fabricated Metal Products Plant

In this plant, aluminum, then manufacture materials for buildings, building materials for houses, and exterior building materials are produced. The manufacturing process of this factory includes a coating process and an alumite process. Effluent from these processes is treated by coagulation-sedimentation.

Figure 6 shows the WET results. The chronic toxicity on cladocerans was consistently high throughout a 2-year survey (TUc > 160). Phase I TIEs were conducted on this sample. The TIE results indicated that chronic toxicity as well as acute toxicity to *C. dubia* was removed by EDTA addition (Fig. 7a) and cation exchange resin treatment (Fig. 7b). These TIE findings suggested

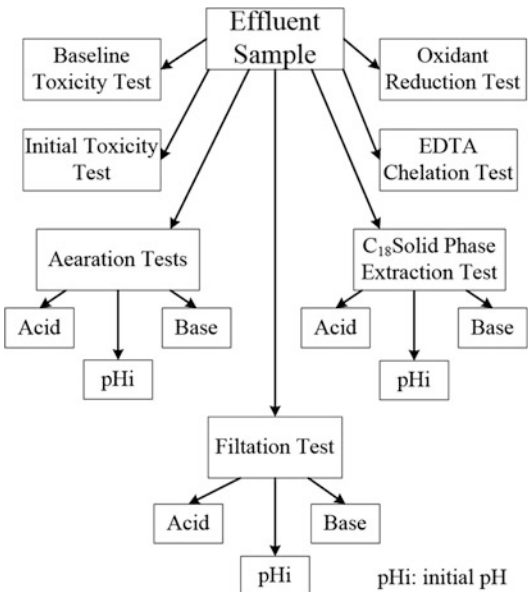

Fig. 5 PhaseI Toxicity characterization tests

Table 4
Effluent manipulation for TIE

Manipulation	Characterization of causative substances
EDTA addition test (3 mg/L)	Heavy metals
Sodium thiosulfate test (10 mg/L)	Oxidants (i.e., chlorine)
pH adjustment test (pH 6.5, 8.5)	Acids or bases
Post C_{18} SPE column test	Non or low polar organic substances
Post anion exchange column test	Anions
Post cation exchange column test	Cations

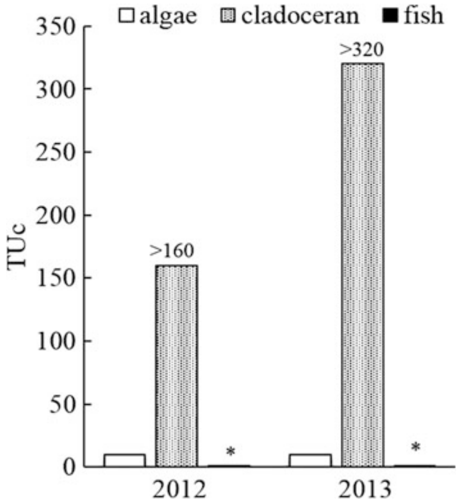

Fig. 6 WET results of fabricated metal products plant #1. *: TUc < 1.25

that metals were responsible for the toxicity. From the results of the ICP-MS analysis, nickel was detected as high as 581 μg/L. The EC20 for *C. dubia* reproduction of nickel were between less than 3.8 μg/L to 6.9 μg/L in the range of hardness of 50–253 mg/L [16]. In our laboratory, NOEC of 1 μg/L was obtained.

Hence, it was suggested that 0.625% of effluent containing nickel of 3.6 μg/L produced chronic toxicity on cladocerans. The contribution of other detected metals such as copper and zinc was negligible.

5.2 Chemical and Allied Products Plant

This factory produced pharmaceuticals and pesticides. After pH adjustment, the effluent was treated by an activated sludge process.

The results of WET tests showed that this effluent was moderately toxic to all test organisms, especially algae and cladocerans (Fig. 8). Phase I TIEs with the *C. dubia* reproduction test were

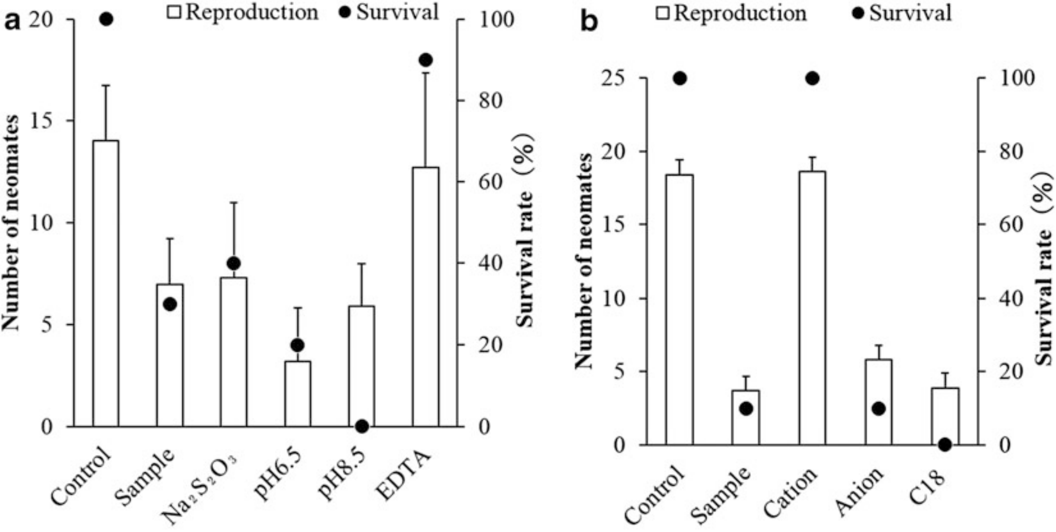

Fig. 7 (**a**) Results of TIE on 0.625% sample (plant#1).Data are presented as mean ± standard deviation ($n = 10$). (**b**) Results of TIE on 0.625% sample (plant#1). Data are presented as mean ± standard deviation ($n = 10$)

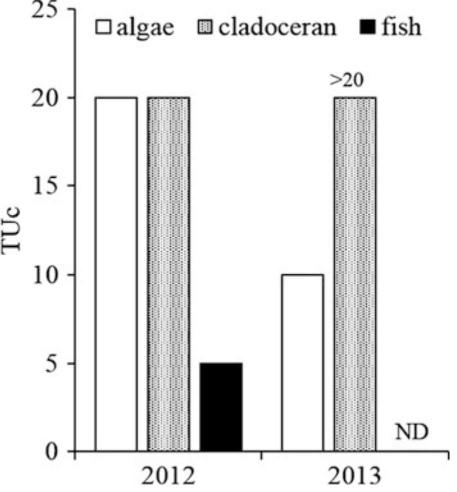

Fig. 8 WET results of chemical and allied products plant #2. *ND* not determined

conducted (Fig. 9a, b). Only pH adjustment to pH 6.5 slightly reduced the chronic and acute toxicity. This finding suggested that base compounds, such as ammonia, might be responsible for the toxicity although further TIEs were not conducted.

This effluent contained T-N of 43.2 mg/L. Based on the former study [11], ammonia-nitrogen was estimated as ca. 40 mg/L. It is well known that ammonia toxicity on aquatic organisms is pH-dependent, and the unionized-ammonia (i.e., NH_3) increases with an increase in pH, resulting in a corresponding

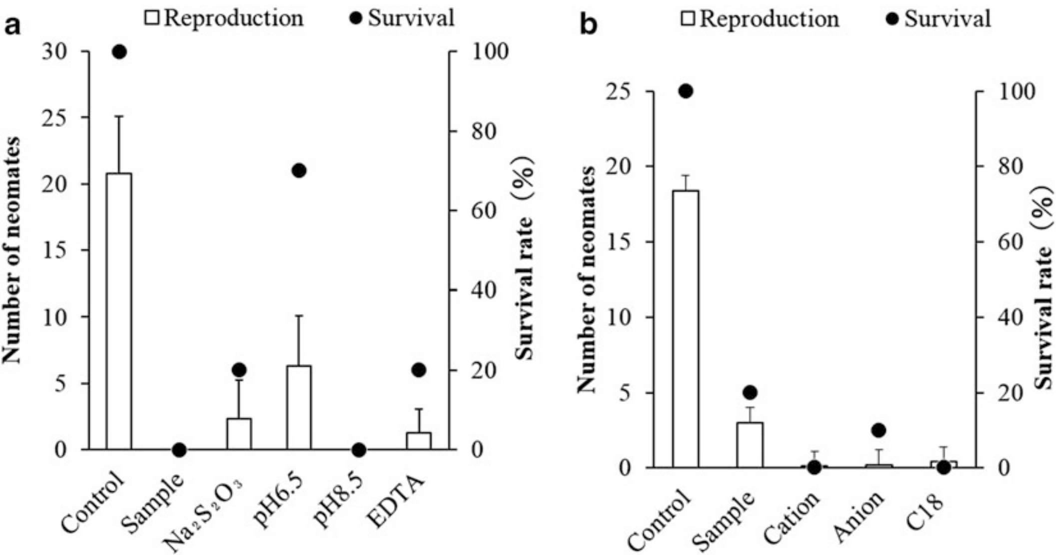

Fig. 9 (**a**) Results of TIE on 20% sample (plant #2). Data are presented as mean ± standard deviation ($n = 10$). (**b**) Results of TIE on 20% sample (plant #2). Data are presented as mean ± standard deviation ($n = 10$)

increase in toxicity. Calculated by Emerson's equation below [17], unionized ammonia concentration in the effluent was ca. 6 mg/L.

$$\text{pKa} = 0.09018 + 2729.92/(273.2 + T)$$

$$\text{f}(\text{NH}_3) = 1/\left(1 + 10^{\text{pKa}-\text{pH}}\right)$$

pKa: acid dissociation constant, T: temperature (°C), f (NH_3): fraction of unionized ammonia (NH_3) to total ammonia

In the effluent sample (20%), concentrations of unionized ammonia were estimated to be 0.02~0.06 mg/L, 0.86 ~ 0.98 mg/L, respectively. The IC25 and IC50 of unionized ammonia ion reproduction of *C. dubia* at pH 8.59 were reported to be 0.35, 0.88 mg/L respectively [18]. Therefore, unionized ammonia was high enough to be toxic at pH 8.5, but not at pH 6.5. The results of TIE testing suggested that toxicants other than ammonia might be involved in apparent toxicity because anion and cation exchange resin treatment did not alleviate the toxicity.

5.3 Drugs and Medicines Manufacturing Plant

This factory produced pharmaceutical intermediates. The process effluent was treated by an activated sludge system and then mixed with used cooling water.

WET results showed chronic toxicity only on cladocerans (Fig. 10). Phase I TIEs results show that cation/anion exchange and C18 solid extraction treatment removed the toxicity (Fig. 11). In addition, the C18 elute showed toxicity. These findings suggested that cations, anions, and organic substances were likely responsible for the observed toxicity. Phase II TIE with GC/MS analysis is necessary to identify the causative agents.

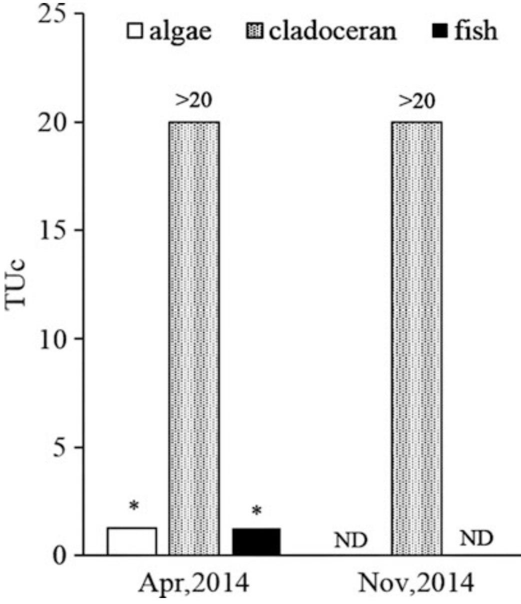

Fig. 10 WET results of drug and medicine manufacturing plant #3.*: TUc < 1.25. *ND* not determined

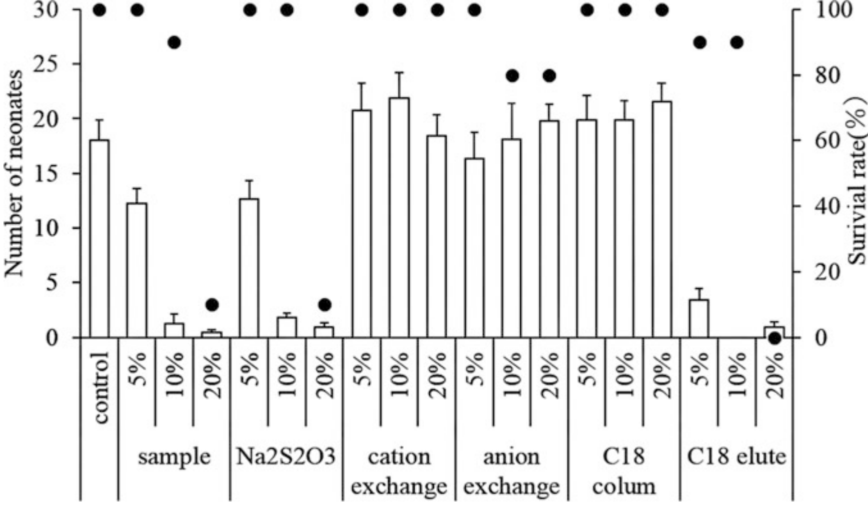

Fig. 11 Results of TIE on 5–20% of sample (plant #3): Data are presented as mean ± standard deviation ($n = 8$)

5.4 Miscellaneous Manufacturing Plant

The process of this factory included an alumite process and plating process. Effluent was treated by neutralization followed by coagulation and sedimentation.

The results of WET tests show chronic toxicity on cladocerans (Fig. 12). The concentrations of nickel in the samples were 190 and 179 μg/L in 2012 and 2013 respectively. Therefore, it is suspected

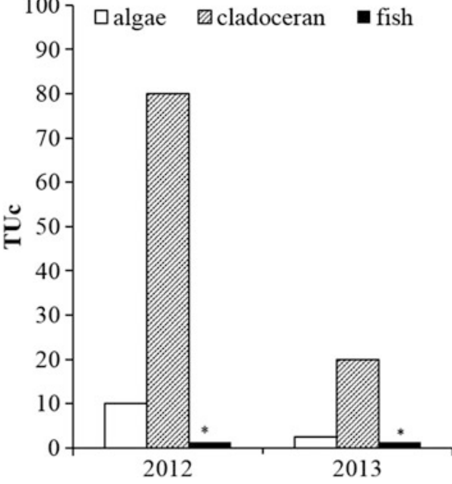

Fig. 12 WET results of miscellaneous manufacturing industry #3. *: TUc < 1.25

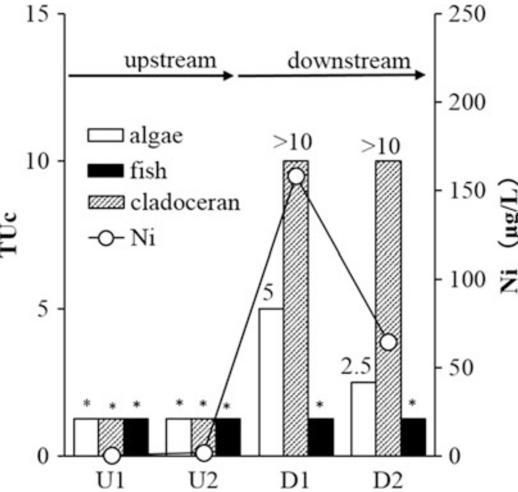

Fig. 13 Results of WET test with receiving water. *: TUc < 1.25

that nickel was the predominant toxicant although TIEs were not conducted.

To investigate the effect of this effluent on receiving water, samples were collected at upstream and downstream sites in the river. After mixing with effluent, the river water samples (D1, D2) showed chronic toxicity on algae and cladocerans although upstream samples (U1, U2) were not toxic (Fig. 13). Concentrations of nickel at downstream D1, D2 were 158, 64.6 μg/L, respectively. Fish toxicity testing did not show any adverse effect due to the difference in sensitivity to this metal. To examine the possibility of source control of nickel discharge, we evaluated the removal efficiency of treatment facilities of four drainage lines that

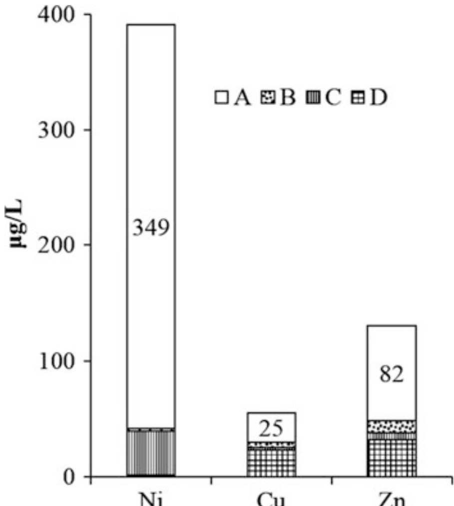

Fig. 14 Contribution of each drain to whole effluent #3

constitute the total effluent. Removal efficiency of copper, zinc, and nickel was evaluated. Average removal efficiency of copper and zinc was more than 98%. However, removal efficiency of nickel in line A was only 13% although those of other lines were more than 98%. Considering the flow ratio of each line, the contribution of each drain on the concentration of final effluent was calculated (Fig. 14). It is clear that the improvement of removal efficiency of line A is essential to reduce the toxicity originating from nickel. The staff of this company confirmed that treatment facilities were getting old and that renewal of these facilities was envisaged.

In this section, four case studies of TIE and TRE are discussed. Further information is available from other sources [19, 20].

6 Alternative Method for WET Testing

The chronic WET tests introduced here require time (>3 days) and are laborious, although they are standardized and sensitive. Furthermore, facilities for culturing the organisms including temperature-controlled chambers and clean bench, equipment such as microscopes and particle counters, and trained personnel are essential for conducting WET tests. That is why specialized consulting firms are conducting conventional WET testing. However, as a tool for routing monitoring on site, culture-free and simple bioassays are required.

To meet these demands, bioassays with bacteria, algae, and invertebrates have been developed. The most well-known example is the bioassay conducted with freeze-dried luminescent bacteria.

After the reactivation of "the reagent," which is the freeze-dried bacteria, the effect on bacteria can be measured by measuring the inhibition of luminescence within one hour [21]. As for invertebrates, a series of cyst-based bioassays have been developed [22]. By using cysts, which are the resting stage of organisms, test organisms can be hatched synchronously within 1 or 2 days and the toxicity tests can then be started with neonates immediately. As for algal tests, the inhibition test with immobilized algae in alginate beads and the delayed fluorescence (DF) assay with cryopreserved algae [23] have been developed. Both types of algae can be stored in a refrigerator for several months, and one can start an assay immediately after the reactivation of the algal cells. The advantages of the latter test are that results can be obtained within 1 day due to the difference of endpoint and results are generally comparable to those of traditional 72 h-inhibition assays [24].

Such bioassays can be useful tools for on-site monitoring. Additional information on small-scale toxicity bioassays is available in other publications [25, 26].

7 Future Prospects

WET testing has been applied successfully in many countries over the last three decades. A variety of acute, sub-chronic, and chronic assays have been developed and standardized. With the increase in number and amount of chemicals potentially discharged into aquatic environment, the importance of bioassay-based effluent management is paramount. Despite these advances, conventional bioassays have intrinsic features that use live organisms, requiring continuous culture and care of test organisms. Some of these shortcomings can be overcome by the use of cysts and cryopreservation. Additionally, another driving force to refine traditional assays comes from animal welfare regulations. Although the zebrafish embryo toxicity assay, which is subject to animal welfare legislation, is used as an alternative for adult fish toxicity test [27], the 3R principles (replacement, reduction, and refinement of animal experiments) will be likely applied to other organisms (i.e., invertebrates) in the future. In this context, the development of alternative methods is certainly called for in the field of ecotoxicology.

Acknowledgments

The authors thank Dr. Christian Blaise for helpful comments on the manuscript.

Glossary

ECx	x percentage of effective concentration
ICx	x percentage of inhibitory concentration
NOEC	No observed effect concentration
TIE	Toxicity identification evaluation
TRE	Toxicity reduction evaluation
TUc	Chronic toxicity unit

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Chapter 18

A Review on Biodegradation and Toxicity Methods: Risk Assessment, Standards, and Analyses

Abdullah M. El Mahdi and Hamidi A. Aziz

Abstract

The role of chemicals in used daily lives is simultaneously obvious and unrevealed. These chemicals are used either in combined form or as some reagents. Around 100,000 different chemical substances find their way into routine products. At 2013, worldwide chemicals industry production was valued at 3.578 trillion dollars. Although no-one would disagree that some chemicals carry significant benefits to humanity—through their use in healthcare, for example—unfortunately some chemicals have harmful impacts on human health and the ecological system and we still don't know enough about their long-term effects. The majority are used without proper tests being carried out on effects of its toxic on health and the environment. Hazardous chemicals are found in the tissue of nearly every individual on Earth and exposure to them has been related to several diseases and the increasing incidence of some of these conditions, and continued exposure to a blend of these chemicals, is alarming. In order to ensure that we are safe, we need chemicals to be properly tested, regulated, and more safety information. This chapter provides a general overview of the biodegradability, standard, and test method and focuses on standards and guidelines for toxicological testing. Some test methods are given. Several methods for evaluating the toxicity and biodegradability of hazardous chemical pollutants have been reviewed in this work. Moreover, it must be noted that biodegradation alone if mostly not enough for acceptance in biological disposal but that also requirements with regard to dissolution and environmental safety are formulated.

Key words Chemical, Environment, Biodegradation, Toxicity, Safety, Standard testing methods

1 Introduction

Human life has continuously involved exposure to chemicals. The substances we eat, drink, and breathe are composed of chemicals. The twentieth century has seen sizable growth in the synthesis of new molecules, some of which have revealed beneficial in treating disease, preserving food, and reducing the cost of produces [1, 2]. The estimates of such substances in the environment range as high as "hundreds of thousands" of chemical structures [3, 4]. In recent decades, there has been widespread concern that synthetic chemical substances increasing in number and concentrations and natural substances may adversely affect human health and

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environment. Unfortunately, some chemicals are damaging wildlife and people, and we still don't know enough about their long-term effects [5]. However, in parallel, the global chemical industry has grown rapidly since 1970. Global chemical output (produced and shipped) was valued at US$171 billion in 1970. By 2010, it had grown to $4.12 trillion [6]. Pesticide use has increased worldwide by 36 fold in the last 45 years (1960–2005) [7]. Global use of pharmaceuticals led to 112% increase in prescription drug sales recorded between 2000 and 2008 [8] and the global use of home and personal care products has increased by 232% and 750% between 1998 and 2013, respectively [9]. Chemicals, and industry are challenged with a fast growing world population, if they are not propyl tested or regulated decreasing durability and quality of life [10].

Nowadays, only a tiny fraction of the chemicals used in the largest volumes has the minimum amount of data publicly available to make an initial basic safety assessment [11, 12]. Some of these chemicals have hazardous properties, they may disrupt hormones or be carcinogenic or persistent, or bioaccumulative or toxic to reproduction. Hazardous chemicals are found in the tissue of nearly every individual on Earth and exposure to them has been linked to several diseases and the increasing incidence of some of these conditions, and continued exposure to a blend of these chemicals, is alarming. In order to ensure that we are safe, we need chemicals to be properly tested, regulated, and more safety information [13]. The natural compounds are more likely to have high biodegradability. In theory, the compounds found in nature, or which are similar, fit into the degradation pathways of natural compounds and should be more degradable. However, that a compound is found in nature does not necessarily mean that it will degrade in all environments—the abundance of the compound as well as where it is found in nature will also play a role [14].

Through growing global environmental awareness, the importance of producing biodegradable materials, whether of a solid or liquid nature, is driving manufacturers to be ahead of the curve and improve market share in the forthcoming. However, uncertainty in establishing confident relationships with laboratories conducting biodegradability assessments spates those not involved daily in this specialty testing. It is clear that the method selected depends on the specific objectives of the test, i.e., the determination of whether a substance is toxic, biodegradable, or treatable. Factors that have to be considered when selecting the test methods are the cost of performing the test, the time and resources involved, and the accuracy required. It often appears that more extensive simulation studies are required after initial screening tests have been performed. Environmental compatibility is usually viewed in respect to biodegradability and toxicity. While the first issue is reached by using a suitable biodegradable base fluid, low toxicity

requires additives that are also environmentally friendly. The estimation of biodegradation rates is an important source of uncertainty in chemical risk assessment. The existing OECD tests for ready biodegradability have been developed to devise screening methods to determine whether a chemical is potentially easily biodegradable, rather than to predict the actual rate, of biodegradation in the environment [14]. In the tests the potential biodegradability is classified usually by evaluating the mineralization of the chemical by measuring the decrease of organic carbon, carbon dioxide evolution, or oxygen demand during the test. In some tests only primary biodegradation is assessed, by analyzing the disappearance of the original parent compound tested (not mineralization). In the OECD regime, a compound is regarded as readily biodegradable if it reaches 60–70% degradation during the 10 day period from the start of the degradation [14]. Biodegradability depends not only on the molecular structure of the test compound but also on the microorganisms available and on other environmental conditions [15]. The standard tests (OECD Test Guidelines and ISO standards) have many arbitrary features compared to actual environments, which compromises their practicability. Standard testing methods for measuring biodegradation in fresh water are described in OECD guidelines (Organization for economic co-operation and development), ASTM standards, ISO standards, etc. The most widely used testing methods for evaluating biodegradation of chemicals in an aerobic aqueous medium are OECD 301 and OECD 310 for ready, and OECD 302 for inherent biodegradability, respectively. Similarly, the International standards ISO 7827, ISO 9408, ISO 9439, ISO 10707, ISO 10708, and ISO 14593 determine the biodegradability of organic compounds in an aerobic aqueous environment and are equivalent to OECD 301 and OECD 310 [16]. Test methods comparable to OECD 302 (inherent biodegradability) were also developed at ISO level (ISO 9887, ISO 9888). The American Standards ASTM D 5271 (plastics) and ASTM D 5864, ASTM D 6139 and ASTM D 6731 (lubricants) address the biodegradability of final products which are soluble, poorly soluble, or insoluble in water. OECD has developed several guidelines for testing of chemicals on biodegradation in an aerobic aqueous medium. As these guidelines are developed for chemicals, these test methods are not always suitable in order to determine biodegradation of complex materials such as fuels and lubricants.

2 Risk Assessment of Chemicals

Over the last few decades, there has been considerable activity in the field of risk assessment. This has mainly taken place in international bodies such as the Organization for Economic Co-operation and Development (OECD), the World Health Organization

(WHO)—especially in the context of its International Programme on Chemical Safety (IPCS)—the European and Mediterranean Plant Protection Organization (EPPO), the Council of Europe and the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) [17, 18]. The European Community has issued various directives and regulations in which risk assessment plays a crucial part [19, 20] and similar activities are taking place in other parts of the world, e.g., the United States, Canada, and Japan. Most of these developments would not have taken place without the contributions of many expert advisory bodies and individual scientists. The production and use of chemicals have begun to threaten biological diversity and ecosystem integrity, and thus humanity‘s very existence [21, 22]. Chemicals are used to make nearly every man-made product and play an important role in the everyday life of people around the world. The chemical industry is the third largest industrial sector in the world, thereby a major economic force. Worldwide, it employs some ten million people and generates billions of euros in shareholder value and tax revenue for governments. Eighty percent of the production takes place in 16 countries, primarily in the OECD member countries. Although chemicals play an important role in products for health and well-being, they may also pose risks to human health and the environment [22]. Risk assessment is a central theme in the control of chemicals [23]. Despite the role of risk assessment as the scientific foundation for man national and international regulatory guidelines, the phrase —risk assessment means different things to different people and is often surrounded by misunderstandings and controversy [22, 24]. Some points of controversy involve the interpretation of scientific studies. Others have to do with science policy issues.

2.1 Risk Management Process

The Risk Management Process consists of a series of steps that, when undertaken in sequence, enable continual improvement in decision-making. Risk comprises impacts on public health and on the environment, and arises from exposure and hazard. Risk does not exist if exposure to a harmful substance or situation does not or will not occur. Hazard is determined by whether a particular substance or situation has the potential to cause harmful effects. The risk management process is initiated by concerns about the risks of particular uses of chemicals or particular situations [22, 25, 26]. Risk assessment and risk management are closely related but different processes, with the nature of the risk management decision often influencing the scope and depth of a risk assessment [27, 28]. In simple terms, risk inspectors ask—How risky is this situation? and risk managers then ask—What are we ready to accept? and—What shall we do about it? Risk assessment is usually seen as the objective/technical part of the process and risk management as the subjective/political part [22, 29]. Risk assessment

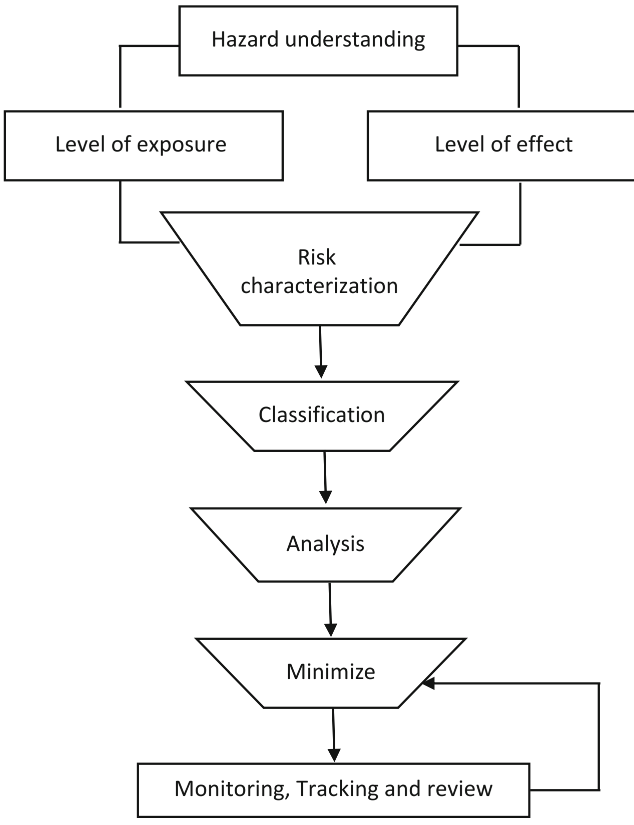

Fig. 1 The eight steps Risk management process

provides information based on the analysis of technical data which describe the form, degree, and characteristics of a risk, i.e., the likelihood of harm to humans or the environment. Risk management is about taking measures based on risk assessments and considerations of a legal, political, economic, and engineering nature. It is mainly a political process, although science is involved in the collecting of technical, social, or economic information [30, 31]. The perfect risk management process consists of eight stages (Fig. 1), in which stages 1–4 belong to the risk assessment phase, while stages 5–8 are in the field of risk management.

1. Hazard understanding (stage 1).
2. Level of Exposure (stage 2).
3. Level of Effects (stage 3).
4. Characterization (stage 4).
5. Classification (stage 5).
6. Analysis (stage 6).
7. Minimization (stage 7).
8. Monitoring, tracking and review (stage 8).

In general risk or hazard assessment, and in particular aquatic hazard classification, are normally based on data obtained in standardized tests for ready biodegradability, but results of tests simulating the biodegradation in water, aquatic sediment, and soil may also be used for these purposes [32, 137]. In order to assess the environmental risk of particular chemicals, information allowing the estimation of its likely concentrations in the environment is required. Degradation of organic chemicals in the environment influences exposure and, hence, it is a key parameter for estimating the risk of long-term adverse effects on biota. Degradation rates, or half-lives, are preferably determined in simulation biodegradation tests conducted under conditions that are realistic for the particular environmental compartment. Simulation tests aim at simulating actual environmental conditions such as redox potential, pH, temperature, microbial community; concentration of test substance and occurrence and concentration of other substrates [33–36, 137]. These are important factors that determine the environmental degradation of organic chemicals in combination with the fundamental properties of the chemical.

2.2 Intelligent Testing Strategies

Intelligent or Integrated Testing Strategies (ITS) are a significant part of the solution to the challenge of carrying out hazard and risk assessments on large numbers of chemicals (ITS) are integrated approaches comprising multiple elements aimed at speeding up the risk assessment process while reducing costs and animal tests [22, 37, 38]. While the details of the different proposals for intelligent testing vary, a number of common components can be identified [22]:

1. Chemical categories and read-across.
2. SARs and QSARs.
3. Thresholds of toxicological concern (TTCs).
4. Exposure-based waiving.
5. In vitro methods.
6. Optimized in vivo tests.

The six ITS components can be subdivided into two main categories: testing approaches (components 5 and 6) and non-testing approaches (components 1–4). The toxicological information can be derived from: (1) chemical categories or read-across, (2) estimation methodologies such as SARs and QSARs, or (3) TTCs, threshold values for chemicals below which no significant risks are expected. If this basic hazard information of the chemicals is combined with adequate exposure information about the chemical, exposure-based waiving (4) can be applied. In vitro methods (5) and optimized in vivo testing (6) can also make significant contributions to the goals of ITS in obtaining reliable

information on the (toxic) properties of chemicals with minimal use of animals. Chemical categories, read across or analogue approaches, (Q)SARs and TTCs have the following characteristics:

- They are all based on the notion that similar compounds (usually structurally similar chemicals) have similar activities.
- They are used to predict properties of interest for (groups of) chemicals for which no or limited data exist.
- Fundamental to all (and sometimes quite limiting) are the size and the quality of the databases on which the methods were based. As with alternative test methods, non-testing methods rely heavily on the availability of in vivo information, i.e., the availability of high quality experimental toxicological data. The availability of high quality in vivo information can be a limiting factor both in the development of in silico and in vitro methodologies. There are clear differences among the components of ITS in terms of financial investments and the time needed to develop and implement them for regulatory application. There are also multiple ways to obtain hazard and risk information by means of different combinations of these components. Read-across and category approaches are probably the simplest tools to reduce animal testing under the REACH legislation but guidance for their regulatory application needs to be developed further. Within the REACH framework, but also within OECD, there is understanding that for reasons of animal welfare, costs, and logistics, it is important to limit the number of tests to be conducted. The goal of Integrated Testing Strategies (ITS) is to increase the use of non-testing information for regulatory decision making of chemicals, and to effectively reduce animal testing without increasing the overall uncertainty [39–41]. In read-across, one or more properties of a chemical of interest are inferred by comparison to a similar chemical or chemicals, for which the properties of interest are known. These properties may include physicochemical properties, environmental fate, toxicity, and ecotoxicity [38, 42].

The read-across can be qualitative or quantitative:

1. Qualitative read-across can be regarded as an application of SAR. The process involves: (a) the identification of a chemical substructure that is common to the two substances (which are therefore analogues) and (b) the assumption that the presence (or absence) of a property/activity for a substance can be inferred from the presence (or absence) of the same property/activity for an analogous substance. This assumption implies that analogues behave qualitatively similarly, and is usually the result of an expert judgment evaluation.

2. Quantitative read-across involves the identification of a chemical substructure that is common to the two substances (which are therefore analogues), and the assumption that the known value of a property for one substance can be used to estimate the unknown value of the same property for another substance. This assumption implies that the potency of an effect shared by different analogous substances is similar, and is also usually the result of an expert judgment evaluation [43, 44].

A chemical category is a group or—family of chemicals whose physicochemical, toxicological, and ecotoxicological properties are likely to be similar or follow a regular pattern as a result of structural similarity [22, 38]. Within a category, different members may be selected for the endpoint desired. If the available test results show that the chemicals in a category behave in a similar or predictable manner, then interpolation and/or extrapolation may be used to assess the chemicals instead of conducting additional testing. Chemical categories are designed on the basis of scientific considerations, including SAR, QSAR, and read-across [45]. Structure-activity relationships (SARs) and quantitative structure-activity relationships (QSARs), collectively referred to as—(Q)SARs, are theoretical models that can be used to predict the physicochemical and biological properties of molecules [46, 47]. They are sometimes called—in silico models because they can be applied by using a computer [48]. A structure-activity relationship (SAR) is a (qualitative) association between a chemical substructure and the potential of a chemical containing the substructure to exhibit a certain biological effect (e.g., a toxicological endpoint).

A quantitative structure activity relationship (QSAR) is a mathematical model that relates a numerical measure of chemical structure (e.g., a physicochemical property) to a physical property or to a biological effect (e.g., a toxicological endpoint) [49]. Over the past 25 years, many OECD member countries have established (Q)SAR tools to provide exposure and effects inputs in ranking and prioritization schemes for in vivo screening and testing programs [50–52]. Thresholds of toxicological concern (TTCs) are exposure threshold values for chemicals below which no significant risk to human health and/or the environment is expected to exist [53]. The establishment of TTC is based on the analysis of toxicological and/or structural data of a broad range of different chemicals. The TTC concept has been proposed as a tool in risk assessment both for defining when no testing is needed based on exposure information and as a substitute for effects values both for human health and the environment [54, 55]. The Threshold of Toxicological Concern (TTC) concept had originally been developed for risk assessment of lifelong and daily oral intake of chemicals, proposing exposure thresholds below which risk to human health is negligible [56–59]. In the early nineteenth century

organic chemicals for which aquatic ecotoxicity was available have been classified into 4 different classes based on their chemical structures assuming different modes of action (MOA) [22, 60]. Verhaar et al. [61] distinguished the following four classes:

Class I: inert chemicals (baseline toxicity) are chemicals that are not reactive when considering overall acute effects and that do not interact with specific receptors in an organism. The MOA of such compounds in acute toxicity is called (lethal) narcosis. Effect concentrations for a number of endpoints can be predicted using QSARs.

Class II: relativity inert chemicals, less inert chemicals are slightly more toxic than predicted by baseline toxicity estimations. These chemicals are often characterized as compounds acting by so-called polar narcosis, and can commonly be identified as possessing hydrogen bond donor acidity, e.g., phenols and anilines [62, 63].

Class III: reactive chemicals display an enhanced toxicity that is related to the phenomenon that these chemicals can react unselectively with certain chemical structures.

Class IV: specifically acting chemicals exhibit toxicity due to (specific) interactions with certain receptor molecules (specific or receptor toxicity). This categorization scheme does not include metals, inorganics, and ionizable organic chemicals.

The exposure to a chemical (or a group of chemicals) can be predicted or measured adequately and the toxicological effects (e.g., based on experimental data or reliable estimates) are much lower than the (predicted) exposure concentration, further animal testing could be waived. In this concept, a decision to waive (exposure-based waiving) or trigger the generation of effects information (exposure-based testing) is a risk-based process for which the exposures for all applications and use scenarios need to be assessed. The application of exposure-based waiving requires extensive, highly detailed exposure information and should provide sufficient information to enable a thorough and reliable assessment of exposure throughout the life cycle [22, 39, 64]. Further development of this approach is part of the current work on the REACH Implementation Projects (RIPs) coordinated by the ECB. The approach is promising, especially when combined with chemical categories, read-across, (Q)SAR, and TTC approaches [22, 65]. In vitro tests include subcellular fractions, as well as cell and tissue cultures maintained for varying periods of time. The development of in vitro tests for different endpoints is being carried out by a wide range of research activities, and different methods are at different stages of standardization and documentation [51, 64]. A number of stand-alone in vitro tests have been adopted by the OECD as official test guidelines. Full replacement of in vivo

procedures is still, however, limited. OECD in vitro test guidelines have been used for many years to evaluate several genotoxicity endpoints and recently, guidelines for in vitro tests on phototoxicity, skin corrosion, and skin absorption were adopted [66]. In these cases, in vitro methods may be used to replace the animal testing currently required for hazard and risk assessment [4]. In the coming years, the advent of new—omics technologies could dramatically increase the synergy between QSAR and in vitro assay methods [42]. Toxicological testing aims to predict possible adverse effects in humans when exposed to chemicals. Currently, it is extensively based on animal testing to identify hazards and the dose-response relationships of chemicals. Ethical concerns have been raised by the use of laboratory animals [67]. However, independent of ethical concerns, the primary objective of the risk assessment of chemical exposures is the protection of human health, wildlife, and ecosystems [68, 69].

3 Toxicity Testing

Research into the toxic effects and testing of substances on humans can be traced back to the early centers of civilization in Egypt, Greece, and China, where toxic chemical substances were used as poisons and sometimes as medicines. Toxicology is the scientific discipline involving the study of actual or potential danger presented by the harmful effects of substances in living organisms and ecosystems, of the relationship of such harmful effects to exposure and of the mechanism of action, diagnosis, prevention, and treatment of intoxications [70, 71]. The science of human toxicology includes both the production and gathering of toxicity data in biological systems, and the subsequent evaluation and interpretation of these data, with the aim of predicting possible risk, or lack of risk, to humans. Toxicity testing is mandatory and the scope depends on the anticipated use. The toxicity testing of environmental chemicals initially focused on determining safe levels of human exposure to toxic chemicals [72]. This testing has now expanded from simple acute and sub-acute tests to careful consideration of data on acute, sub-acute, and chronic toxicity, specific toxicity such as carcinogenicity, mutagenicity, reproductive toxicity, and, more recently, immunotoxicity, neurotoxicity, dermal toxicity and other organ tests. In addition to these toxicity studies, data on the mechanisms of action at the tissue, cellular, subcellular, and receptor levels, as well as toxic kinetic data, greatly facilitate the interpretation of toxicity data and the assessment of the potential hazard to humans. Protocol toxicology and receptor toxicology are essential to provide the optimum framework for risk prediction [22, 73–76] and this requires international management.

The large number of chemicals involved require rules to be able to select priority chemicals and testing strategies. This is because of the time and cost that testing requires as well as for animal welfare reasons. Such testing strategies increasingly include basic steps that rely on alternative estimation methods, such as quantitative structure-activity relationships (QSARs), structure-activity relationships (SARs), and in vitro tests, rather than on immediate testing on experimental animals. The starting point of such strategies should be the regulatory information requirements [22]. Developments in molecular biology and biotechnology are paving the way for major improvements in how scientists evaluate the health risks posed by potentially toxic chemicals found at low levels in the environment. These developments would make toxicity testing more rapidly, less costly, and more directly appropriate to human exposures [77, 78]. This resulted in a major perfect shift for toxicity testing—from apical endpoints in animal-based tests to mechanistic endpoints through description of pathways of toxicity (PoT) in human-based cell systems. Toxicology evaluation of chemicals is poised to take advantage of the on-going revolution in biology and biotechnology. This revolution is making it increasingly possible to study the effects of chemicals using cells, cellular components, and tissues—preferably of human origin—rather than whole animals. While in the past, approach has led to a testing system that is lengthy and costly and that uses many animals [79, 80]. The target of toxicity testing should be the collection of appropriate outcomes from test systems in order to assess the prospective risks posed to human populations at ambient exposure levels; i.e., provide the data inputs necessary for an accurate assessment of human risk [81].

Toxicology testing is a common requirement when making label claims for environmentally acceptable products or materials, such as environmentally acceptable lubricants. It is highly recommended to review requirements with the appropriate regulatory agency to determine which methods are required for label claims and regulatory acceptance. Commonly requested OECD methods for toxicology testing include OECD 201, OECD 202, and OECD 203.These standard methods test against a range of organisms including Algae and cyanobacteria, Daphnia species, and Freshwater fish species [82]. The OECD guidelines are the most commonly used tests for assessing ecotoxicity of materials in aquatic environment. They comprise 11 internationally agreed testing methods for environmental effects. Tests following the OECD guidelines are useful for both risk assessment and classification purposes. These guidelines describe test methods designed to evaluate the aquatic toxicity of chemicals. The toxic effect on different types of freshwater species can be evaluated: (1) algae, (2) plants, (3) invertebrates, and (4) fish [83]. Thru the issue of the NRC report Toxicity Testing in the twenty-first century: A Vision and a Strategy in

2007 toxicity testing reached a revolving point. The report established forth a vision for transforming conventional toxicity testing by integrating advances in systems biology, epigenetics, toxicogenomics, bioinformatics, and computational toxicology. The new system that was described in the report would be based primarily on in vitro methods that can be used to evaluate changes in biological processes with cells, cell lines, or cellular components, preferably of human origin. The motivation for the new system was to accomplish four strategy objectives: (1) to provide broad coverage of chemicals, chemical mixtures, results, and life stages, (2) to reduce the cost and time of testing, (3) to use fewer animals and cause slight suffering in the animals used, and (4) to develop a stronger scientific basis for assessing health effects of environmental agents [77].

3.1 Chemical Characterization

Chemical characterization is intended to provide insights into key questions, including a compound‘s stability in the environment, the potential for human exposure, the likely routes of exposure, the potential for bioaccumulation, possible routes of metabolism, and the likely toxicity of the compound and possible metabolites based on chemical structure or physical or chemical characteristics. A variety of computational methods might be used to predict those properties and characteristics. After chemical characterization, decisions might be made about what further testing is required or whether it is needed at all. In most cases, chemical characterization alone is not expected to be sufficient to reach decisions about the toxicity of an environmental agent. Advances in molecular biology, biotechnology, and other fields are paving the way for major improvements in how scientists evaluate the health risks posed by potentially toxic chemicals found at low levels in the environment. These advances would make toxicity testing quicker, less expensive, and more directly relevant to human exposures [76].

3.2 Toxicity Testing of Chemicals

In many countries, a framework of OECD guidelines [83] rules the process of toxicity testing of environmental chemicals. Hundreds of new chemicals, which are used among others as industrial chemicals, pesticides, food additives, biotechnology products, and pharmaceuticals, reach the world market each year and may require safety testing in most parts of the world. In addition, regulations exist or are under way, both at national and international levels, that call for (additional) testing and assessment of chemical substances already on the market [84]. The toxicity of a substance depends on three factors: its chemical structure, the extent to which the substance is absorbed by the body, and the body's ability to detoxify the substance and eliminate it from the body [85]. Toxicity tests have taken on increased importance after scientists realized that many substances are toxic to living things at levels below chemical detection limits and that there are no methods to analyze for many

toxic substances [86]. The Biology Laboratory conducts an array of toxicity bioassays. A toxicity bioassay may be run as a screening test, where the toxicity of a sample is compared to that of a control water, or as a definitive test, where several portions of the sample are diluted with varying amounts of the control water and their results compared to the control water [87]. The screening tests indicate whether toxicity is present in the sample. The definitive tests indicate the amount of toxicity presented by the sample. Additionally, the results of a toxicity bioassay may be measured as either an acute response such as mortality or a chronic response such as growth or reproductive effects. Toxicity must be assessed by several bioassays and different test organisms, for a reliable determination of the environmental impact of the sample [88, 89].

3.3 Bioassay Test for Toxicity

Up until as recently as 1986, canaries were used in coal mines to warn miners of the presence of harmful gases such as carbon monoxide or methane. Since the birds are more sensitive to these gases than humans, they are affected before the gases get to a level that is dangerous for humans [90, 91]. This use of a living organism to test for toxicity is called a bioassay. Bioassays can provide a measure of the whole-effect, produce for a complex mixture integrating different factors, such as pH, solubility, antagonism or synergism, bioavailability, etc. The biological response induced by a substance in different test organisms is different. The use of a battery of bioassays involving different species at different trophic levels is an efficient and essential tool for predicting environmental hazards to the aquatic ecosystem [92–94]. Micro bioassays using bacteria or enzymes are increasingly applied to measure chemical toxicity in the environment. Attractive features of these assays may include low cost, rapid response to toxicants, high sample throughput, modest laboratory equipment and space requirements, low sample volume, portability, and reproducible responses. Enzymatic tests rely on measurement of either enzyme activity or enzyme biosynthesis [95, 96]. Dehydrogenases are the enzymes most used in toxicity testing. Assay of dehydrogenase activity is conveniently carried out using oxidoreduction dyes such as tetra zolium salts. Other enzyme activity tests utilize ATPases, esterases, phosphatases, urease, luciferase, beta-galactosidase, protease, amylase, or beta-glucosidase. Recently, the inhibition of enzyme (beta-galactosidase, tryptophanase, alpha-glucosidase) biosynthesis has been explored as a basis for toxicity testing [97]. Enzyme biosynthesis was found to be generally more sensitive to organic chemicals than enzyme activity. Bacterial toxicity tests are based on bioluminescence, motility, growth, viability, ATP, oxygen uptake, nitrification, or heat production. An important aspect of bacterial tests is the permeability of cells to environmental toxicants, particularly organic chemicals of hydrophobic nature [98]. The names of the assays and their basis are: Microtox (bioluminescence), Polytox (respiration), ECHA

Biocide Monitor (dehydrogenase activity), Toxi-Chromotest (enzyme biosynthesis), and MetPAD (enzyme activity) [97, 98]. An important feature common to these tests is the provision of standardized cultures of bacteria in a freeze-dried form. Two of the more recent applications of microbioassays are in sediment toxicity testing and toxicity reduction evaluation.

3.4 In Vivo Versus In Vitro

Historically, regulatory requirements on chemical risk assessments are largely based upon in vivo toxicity testing of individual substances on "representative" single species, supported by some in vitro and in silico approaches. In vivo studies are very important in both the field and the laboratory (for validation), they are based on a wide variety of end points, including cell differentiation and enzyme activities. However, it is not possible to use in vivo methods for routine or monitoring studies: ethical problems, expensive, time consuming, and big installations are needed. In vitro bioassays can be performed more quickly, these tests are much more cost effectives than in vivo assays. However, in vitro assays are not able to explain all the mechanisms. Almost all they are using in vitro systems to evaluate possible genetic toxicity and cytotoxicity as well as screening to predict organ toxicity or to understand related molecular mechanisms [99–101]. High-throughput screening (HTS) assays that measure the in vitro toxicity of environmental compounds have been widely applied as an alternative to in vivo animal tests of chemical toxicity. Current HTS studies provide the community with rich toxicology information that has the potential to be integrated into toxicity research. With its low cost and short testing time, HTS has been viewed as the potential alternative to animal models. In contrast with virtual screening techniques (e.g., QSAR or docking), HTS does not require prior knowledge about potential hits or

3D structures of involved molecular targets. HTS is a process that screens thousands to millions of compounds using a rapid and standardized protocol. Recent HTS techniques are usually combined with robotic systems.

Compared to the limited amount of historical animal toxicity data, the chemical–response data space obtained from HTS is much more complex and keeps growing daily [102].

4 Biodegradation Testing

Biodegradation is defined as the breakdown or mineralization of an organic material due to microbial activity. Due to the large differences between environments, a biodegradability claim corresponds to a specific well-defined environment. Therefore, test methods, which determine the biodegradability of a material, always refer to a specific environment, i.e., fresh water, marine water, soil, or

compost and aerobic or anaerobic conditions [103]. Biodegradation tests have their roots in the development of degradable synthetic surfactants in the mid-seventies. The first guidelines were developed by the Organization for Economic Cooperation and Development (OECD) in 1981. CEN (European committee for standardization), ISO (International Organization for Standardization), and ASTM (American Society for Testing and Materials) also developed biodegradability testing methods for organic chemicals and also for more complex materials [104, 105]. The majority of the standards for testing biodegradation in fresh water were developed for—chemicals or—organic compounds, while a few standards are especially developed for plastics and lubricants. No specific standards for solvents are currently available. Because of the large number of chemicals that are being used in society an approach is required, which provides adequate knowledge for decision making as regards environmental protection, but which at the same time enables costs for testing to be kept to a minimum. Ideally, a system is required that allows preliminary screening of chemicals, using relatively simple tests of ultimate biodegradability, with the identification of those chemicals for which more detailed, and hence costlier, studies are needed. Biodegradability of a novel chemical is typically assessed using a standardized protocol. A diverse set of established protocols can be used to classify a chemical as readily biodegradable (e.g., OECD, 2006; OECD 309; ASTM 5988; ISO 14593; reviewed in Coleman and Gather [106, 107]). Chemicals that achieve this standard are assumed to biodegrade during their residence time in a WWTP and are categorized as being low risk aquatic pollutants [108, 109].

4.1 Biodegradability Testing

Taking benefit from experience gained during the design of a two-tiered test system for surfactants consisting of Screening and Confirmatory [110, 111], the experts published a test system in 1981 that was meant to evaluate the biodegradability of industrial chemicals as a property that forms part of their ecotoxicity [111]. This test system is in principle suitable for a wide variety of chemicals and was adopted by the EEC (1984).

The system is also known under the name “OECD-hierarchy” as three different levels are distinguished [22, 112, 113]:

1. Ready Biodegradability (RBT) for quickly selecting “soft” chemicals in order to avoid time and money consuming further research [114, 140]. Conducted under aerobic conditions, in which a high concentration of the test substance (in the range of 2–100 mg/L) is used and biodegradation is measured by nonspecific parameters like Dissolved Organic Carbon (DOC), Biochemical Oxygen Demand (BOD), and CO_2 production.
2. “Inherent Biodegradability” (IBT), to demonstrate the potential degradability of a compound. Aerobic tests that possess a

high capacity for degradation to take place, and in which biodegradation rate or extent is measured. The test procedures allow prolonged exposure of the test substance to microorganisms and a low ratio of test substance to biomass, which offers a better chance to obtain a positive result compared to tests for ready biodegradability [115–117].

3. "Simulation" (ST), designed to measure the rate of biodegradation in a specified environmental compartment. Aerobic and anaerobic tests that provide data for biodegradation under specified environmentally relevant conditions. These tests simulate the degradation in a specific environment by use of indigenous biomass, media, relevant solids (i.e., soil, sediment, activated sludge, or other surfaces) to allow sorption of the chemical, and a typical temperature which represents the particular environment [118, 137].

4.2 Assessing Biodegradability and Biodegradation Rates

The estimation of biodegradation rates is an important source of uncertainty in chemical risk assessment. The existing OECD tests for ready biodegradability have been developed to devise screening methods to determine whether a chemical is potentially easily biodegradable, rather than to predict the actual rate, of biodegradation in the environment [118, 119]. Estimated rate constants of degradation processes, particularly for biodegradation, generally, have larger margins of uncertainty than those of exchange processes. In principle, there are two approaches to obtaining biodegradation rate constants for a particular compartment:

1. A theoretical approach, making use of QSARs. (Quantitative) Structure-Activity Relationships [(Q)SARs] are methods for estimating properties of a chemical from its molecular structure and have the potential to provide information on hazards of chemicals, while reducing time, monetary cost, and animal testing currently needed. QSARs are employed as scientifically credible tools for predicting the acute toxicity of chemicals when few, or no, empirical data are available [120].
2. An experimental approach, on the basis of standardized test results.

   Most biodegradability data are derived from standard methods that make use of some artificial environment accommodating water, auxiliary nutrients, the test chemical, and an inoculum [22, 118].

   The first biodegradability tests were the result of legislation on detergents that came into force soon after the introduction of synthetic surfactants in the early 1960s. A common system was published by the OECD in 1976 [121, 122]. It consists of two stages that differ both in principle and in the conclusions

that can be drawn from them. These tests are suitable for anionic and nonionic surfactants:

(a) The OECD screening test (OST) is a static flask test which is relatively quick and simple to carry out. This test should be considered an acceptance test, not as a rejection test. It selects soft surfactants that do not have to be tested further because high biodegradability is expected in sewage treatment plants.

(b) The OECD confirmation test (OCT) is based on a simulation of the conditions existing in an activated sludge plant. This test should be used for any surfactant which may not have passed the OST, either to confirm or disprove the first results obtained.

In recent decades, the OST and OCT have been modified and other methods have also been added by the OECD. This was done to design a three-tier test system to evaluate the biodegradability of industrial chemicals as a property that is part of their ecotoxicity [123].

4.3 Microbial Ecology in Biodegradation Testing

Biodegradation tests play the key role in chemical persistence assessment upon which environmental risk assessment is performed. Current OECD tests have been designed in the manner that does not consider microbial inocula and there is little understanding of chemical breakdown pathways and how they relate to the complexity and variability of environmental compartments [37, 124]. Microbial community characterization helps to better understand the microbiology of samples that are used as inocula in biodegradability testing and their potential to degrade a given chemical. Optimization of microbial inocula could also help to reduce the costs of fate studies and risk assessments by increasing the probability of biodegradation and decreasing the rate of test failures, by increasing the reproducibility of fate studies. Current OECD tests are conducted with variable inocula and there is no inoculum standardization prior to biodegradability testing, which has been identified as the key factor affecting the reproducibility of chemical fate studies [115, 125]. Molecular omics‘ tools are increasingly affordable, commonly used methods, which could be used alongside traditional OECD methods to study chemical biodegradation in the environment. Application of microbial ecology methods also provides the opportunity to study chemical biodegradation pathways and to gain more insights into complex biodegradation processes conducted by microbial communities in a given environment [36].

These methods may be applied to support biodegradation tests by studying the functional diversity of inocula and its potential for biodegradation. Further development of such approaches could

potentially identify the scope for adaptation within communities to degrade chemicals. Chemical fate is associated with proliferation of degrading bacteria which often leads to changes in bacterial community composition. Changes occurring in microbial populations during biodegradation of chemical, in particular the abundance and diversity of specific degraders, can be then correlated with chemical fate. In contrast, lack of changes in bacterial community and low abundance of specific degraders coupled with lack of chemical biodegradation may suggest potential persistence of given chemical [36]. Microorganisms are increasingly used as specific sensing devices for measuring chemical concentrations in the environment. These biosensors measure gene expression induced by the presence of a chemical of interest, which serves as a measure of its bioavailable concentration [126]. Most screening and simulation OECD tests lack environmental realism, and this leads to difficulties with data extrapolation from laboratory to real world scenarios. Hence, the lack of environmental realism is a key challenge for environmental risk assessment and there is the need to develop tools that take account the complexity of exposed environments and enable assessment of site-specific effects [36, 127]. Omics' could also help us to understand interactions between chemicals and environmental factors, e.g., temperature, salinity, organic matter content, and can indicate how environmental factors might modulate microbial responses to chemicals [36, 128].

5 Standard Testing Methods

5.1 OECD Guidelines

In 1987, the member countries of the Organization decided to investigate existing chemicals. In 1991, they agreed to begin by focusing on High production volume (HPV) chemicals, where production volume was used as alternate for data on occupational, consumer, and environmental exposure. Each country agreed to "sponsor" the assessment of a proportion of the HPV chemicals. Countries also agreed on a minimum set of required information, the screening information dataset (SIDS). Six tests are: acute toxicity, chronic toxicity, developmental toxicity/reproductive toxicity, mutagenicity, ecotoxicity, and environmental fate, References [129, 130]. Using SIDS and detailed exposure data OECD's High Production Volume Chemicals Programme conducted initial risk assessments to screen and identify any need for further work. During the late 1990s, the OECD member countries began to assess chemical categories and to use quantitative structure–activity relationship (QSAR) results to create OECD guidance documents, as well as a computerized QSAR toolbox [131]. In 1998, the global chemical industry, organized in the International Council of Chemical Associations (ICCA) initiative, offered to join OECD efforts. The ICCA promised to sponsor by 2013 about 1000

substances from the OECD's HPV chemicals list "to establish as priorities for investigation," based on "presumed wide dispersive use, production in two or more global regions or similarity to another chemical, which met either of these criteria" [132]. OECD in turn agreed to refocus and to "increase transparency, efficiency and productivity and allow longer-term planning for governments and industry." The OECD refocus was on initial hazard assessments of HPV chemicals only, and no longer extensive exposure information gathering and evaluation. Detailed exposure assessments within national (or regional) programmes and priority setting activities were postponed as post-SIDS work.

5.2 OECD Biodegradability Tests

OECD 301 Testing—Ready/Ultimate Biodegradability (Includes OECD 310). The OECD 301 series of tests allows for direct, explicit certification of a material's biodegradability. The methods establish threshold criteria for the direct classification and marketing of materials under the terms of Ready or Ultimate Biodegradability. A material is considered Readily Biodegradable if 60% (or 70% for some tests) of the organic carbon in the material is converted to CO_2 within a 10 day window as well as a full 28 day test period. The 10 day window is defined as beginning when 10% of the organic carbon has been converted to CO_2 and lasts for 10 days (but before 28 full days of the test). A material is considered Ultimately Biodegradable (under the sub classification of Inherent Biodegradability) if 60% (or 70% for some tests) of the organic carbon in the material is converted to CO_2 over the duration of the test. The test can be extended beyond 28 days in this case. OECD 311—Anaerobic Biodegradability of Organic Compounds in Digested Sludge. The OECD 311 methodology is used to assess the biodegradability of organic chemicals in anaerobic conditions. This test method simulates conditions similar to that in an anaerobic digester, which is a biological reactor typically used to digest sewage sludge after water treatment. Residual chemicals that are not degraded in standard aerobic treatment basins will often end up in anaerobic digesters as a last treatment option prior to discharge to the environment [137, 140].

5.3 ASTM Biodegradability Tests

ASTM E1720—Standard Test Method for Determining Ready, Ultimate, Biodegradability of Organic Chemicals in a Sealed Vessel CO_2 Production Test. The ASTM E1720 test method monitors the conversion of organic carbon to CO_2 in a very similar fashion to the OECD 301 series tests. In fact, this method was derived based on OECD 301B and is nearly identical to OECD 310. ASTM E1720 provides explicit language for classification of the material as Readily or Ultimately Biodegradable but does not allow extension of the test beyond 28 days. This test is conducted in closed, gas-tight bottles. ASTM D5210—Standard Test Method for Determining the Anaerobic Biodegradation of Plastic Materials in

the Presence of Municipal Sewage Sludge. The ASTM D5210 test method is very similar to OECD 311 and is used to assess the biodegradability of organic chemicals in anaerobic conditions. This test method simulates conditions similar to that in an anaerobic digester, which is a biological reactor typically used to digest sewage sludge after water treatment. Residual chemicals that are not degraded in standard aerobic treatment basins will often end up in anaerobic digesters as a last treatment option prior to discharge to the environment. ASTM D5271—Standard Test Method for Determining the Aerobic Biodegradation of Plastic Materials in an Activated-Sludge-Wastewater Treatment System.

The ASTM D5271 test method evaluates the aerobic biodegradation of plastic materials in activated sludge wastewater treatment systems. The duration of this test method is a maximum of 6 months. No explicit thresholds or classifications are permitted based on method language for certification purposes. The method is used to index the biodegradability of plastic materials relative to controls that are known to persist (not biodegrade). The standard was withdrawn in 2011. ASTM D5338—Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials Under Controlled Composting Conditions. Incorporating Thermophilic Temperatures. The ASTM D5338 test method evaluates the biodegradation of plastic materials under controlled composting conditions. Controlled composting conditions are considered those conditions where oxygen, temperature, moisture, and pH levels of the compost are maintained at optimal levels. Commercial composting facilities are the relevant disposal compartment for materials that undergo assessments based on this type of test method. The duration of the test can be up to 180 days. No explicit thresholds or classifications are permitted based on method language for certification purposes. ASTM D5511—Standard Test Method for Determining Anaerobic Biodegradation of Plastic Materials Under High-Solids Anaerobic-Digestion Conditions. The ASTM D5511 test method evaluates the anaerobic biodegradation of plastic materials under high solids conditions. This method differs from ASTM D5210 primarily in the moisture content of the inoculums used. The biodegradation is evaluated based on conversion of organic carbon to CO_2 and CH_4, as would be expected in an anaerobic digester. The duration of the test can be extended up to 120 days without significant inoculum activity loss as evidenced in our laboratory. No explicit thresholds or classifications are permitted based on method language for certification purposes.

6 OECD Biodegradation Tests

A range of methods for investigating biodegradation processes have been developed to predict the fate of chemicals in the environment. Most efforts have focused on the fate of chemicals in the aquatic

Table 1
Reference chemicals and their classification into bins. Adapted from [138]

Bin	Description	Half-life (days)	Chemical example
1	Reference chemicals that would normally pass a RBT or modified RBT test	<15	Aniline, sodium benzoate, phenol
2	Reference chemicals that would normally pass an enhanced screening biodegradability test but currently fail any other tests	16–40	4-Chloroaniline,4-fluorophenol, 1,2,3-trimethylbenzene
3	Reference chemicals that would normally fail any biodegradability screening test whether modified RBT or enhanced screening biodegradability test	41–60	*o*-Terphenyl, cyclodecane, dibutylphenol
4	Reference chemicals that should never pass a modified RBT or an enhanced biodegradability screening test	>60	Hexachlorobenzene, benzo (a) pyrene, hexachlorohexane

RBT ready biodegradability tests

environment, especially in wastewater treatment processes [133]. Testing biodegradability under laboratory conditions aims to obtain a reliable prediction of the likelihood of the biodegradability of chemicals in the environment [134]. Over 30 years ago, in 1981, the OECD first published its guidelines for testing the biodegradation of chemicals. There have been several amendments and additions since, including updated methods for assessing ready biodegradability in 1992 and introduction of the CO_2 headspace test [135]. Three groups of tests were defined: (1) ready biodegradability (or screening), (2) inherent biodegradability, and (3) simulation [136, 137]. Regulators and industry with an agreed set of properties and characterized set of biodegradability behavior, which cover a range of environmental persistence and non-persistence [136], have proposed a list of reference chemicals for use as positive and negative controls in standardized biodegradability tests (Table 1). These chemicals group into bins (Table 1), which line up with OECD tiered testing (Fig. 2) and show the relationships between the screening and higher tier tests.

The choice of a test should depend on the testing purpose, and under REACH, the testing is ordered according to production level and properties of a chemical [137, 138]. Ready biodegradability tests (RBTs) are considered a stringent first tier and indicate if a chemical is rapidly degradable or not. Seven methods (Table 2) permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium and they are based on the removal of organic chemicals measured as dissolved organic carbon (DOC) (OECD 301 A, OECD 301 E), the production of the catabolic end product carbon dioxide (OECD 301 B), the determination of

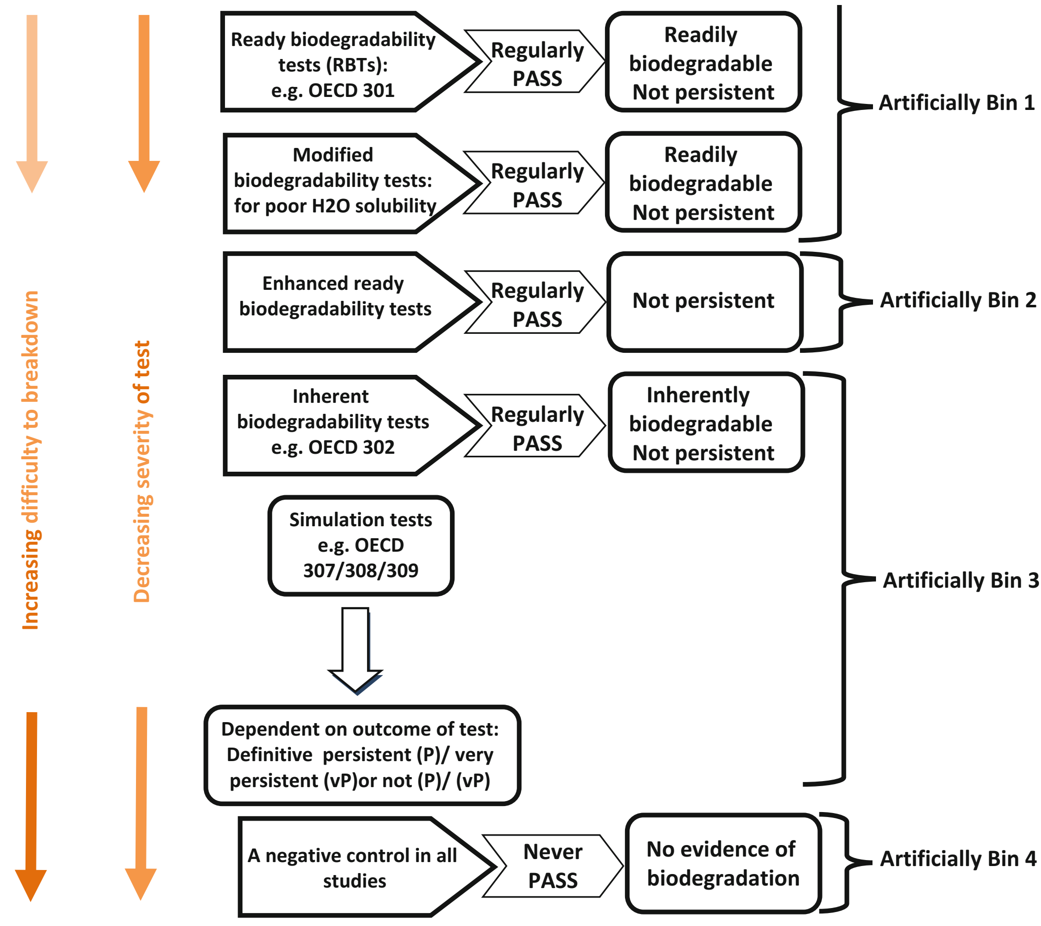

Fig. 2 Linked in between ready biodegradability and simulation tests and the bins

the biochemical oxygen demand (BOD) (OECD 301 C, OECD 301 D, OECD 301 F) [133, 138, 140] and by measuring the inorganic carbon (IC) produced in the test bottles [136].The second tier tests are inherent tests where inherent biodegradability can be measured by specific analysis (primary biodegradation) or by nonspecific analysis (ultimate biodegradation). Inherent biodegradability tests include Modified SCAS Test [139], Zahn-Wellens/EMPA Test [97, 140], and Modified MITI Test (II) [141] (Table 2).

Table 2
Overview of OECD biodegradation tests

Biodegradation test	OECD guideline	Pass level	Incubation conditions	Chemical concentration	Inoculum source	Inoculum size	Test duration
Ready biodegradability tests (screening tests)	OECD 301 A	70% DOC removal	Aerobic	10–40 mg DOC/l	Activated sludge, sewage effluents, surface waters, soils or mixture of these	r 30 mg/l settled sewage; r 100 ml effluent/l	28 Days approx. 10^7–10^8 cells/l
	OECD 301 B	60% $ThCO_2$		10–20 mg DOC/l			
	OECD 301 C	60% ThOD		100 mg/l	Fresh samples from sewage treatment works, industrial WWTPs, soils, lakes, seas, mixed thoroughly together	30 mg/l settled sewage;	approx. 10^7–10^8 cells/l
	OECD 301 D			2–10 mg/l or 5–10 mg ThOD/l	Derived from secondary effluent of WWTP or laboratory-scale unit predominantly domestic sewage, alternatively surface water e.g., river, lake	r 5 ml effluent/l;	approx. 10^4–10^6 cells/l
	OECD 301 E	70% DOC removal		10–40 mg DOC/l	Derived from secondary effluent of WWTP or laboratory-scale unit predominantly domestic sewage	0.5 ml effluent/l;	approx. 10^5 cells/l
	OECD 301 F	60% ThOD		100 mg/l or 50–100 mg ThOD/l	Activated sludge, sewage effluents, surface waters, soils or mixture of these	r 30 mg/l settled; r100 ml effluent/l;	approx. 10^7–10^8 cells/l
	OECD 310	60% ThIC		20–40 mg C/l	Activated sludge, sewage effluents, surface waters, soils or mixture of these	4–30 mg SS/l or 10% v/v secondary effluent	
Inherent (potential) biodegradability tests	OECD 302 A	4 20% ThBOD, ThDOC removal or ThCOD (primary	Aerobic	2–10 mg/l	Mixed settled sludges after two weeks aeration period	A high concentration of Not defined aerobic micro-organisms	
	OECD 302 B	biodegradation; 420% Th BOD, ThDOC removal or		50–400 mg DOC/l	Activated sludge	0.2–1.0 g dry matter/l	
	OECD 302C	ThCOD (ultimate biodegradation)		30 mg/kg	Activated sludge	100 mg/kg	

(continued)

Table 2
(continued)

Biodegradation test	OECD guideline	Pass level	Incubation conditions	Chemical concentration	Inoculum source	Inoculum size	Test duration
Simulation tests	OECD 303 A	Estimation of half-lives for materials exhibiting first-order degradation patterns.	Aerobic/ anaerobic	41–100 μg/l	Activated sludge	2.5 g/l dry matter; 2–10 ml/l 120 Days effluent	
	OECD 303 B				Airborne inoculation, settled sewage	1 ml/l of settled sewage	
	OECD 307	In the absence of first-order kinetics degradation times for 50%, (DT_{50}) and 90% (DT_{90}) may be reported			Representative soil; a sandy loam or silty loam or loamy sand with a pH of 5.5–8.0, organic carbon content of 0.5–2.5%	Microbial biomass of at least 1% of total organic carbon	
	OECD 308				Sediments from sampling sites selected based on the history of possible agricultural, industrial or domestic inputs to the catchment and the waters upstream	Not defined	
	OECD 309				Surface water from sampling sites selected based on the history of possible agricultural, industrial or domestic inputs	Not defined	
	OECD 314 (A–E)				Raw wastewater, activated sludge, anaerobic digester sludge, treated effluent-surface water mix, untreated effluent-surface water mix,	Not defined	

DOC dissolved organic carbon, *ThBOD* theoretical biochemical oxygen demand, *ThCOD* theoretical chemical oxygen demand, *ThCO*$_2$ theoretical carbon dioxide, *ThDOC* theoretical dissolved organic carbon, *ThIC* theoretical inorganic carbon, *ThOD* theoretical oxygen demand, *WWTP* wastewater treatment plant

7 Marine Aerobic Aqueous Environment

7.1 Biodegradation

Due to the growing awareness of the need to protect the marine environment against the increasing loads of chemicals, biodegradation methods were also developed for the marine environment. Biodegradation in a marine aerobic environment differs from biodegradation in a freshwater aerobic environment due to differences with regard to (1) the microbial population and (2) the chemical parameters of the water (salt content, nutrient content, etc.). Currently, biodegradation test methods for a marine environment are developed on OECD level, ISO level, and ASTM level. No European test method has been developed yet [142, 143].

7.1.1 OECD Guidelines

One OECD guideline with regard to the evaluation of the biodegradation of chemicals in seawater is developed yet: OECD 306—Biodegradability in Seawater [143]. The results of this test give a first impression of biodegradability in seawater. If the results are positive (>70% DOC removal or >60% ThOD), it may be concluded that there is a potential for biodegradation in a marine environment.

Although it must be noticed that this guideline is no simulation test as nutrients are added and the test concentration of the substance is much higher than the concentration that would be present in the sea. If a more definitive value would be required for the degree of biodegradation in seawater, other methods need to be used (e.g., simulation test in seawater using a test item concentration closer to the likely environmental concentration). In this guideline two test methods are described: (1) the shake flask method and (2) closed bottle method [144]. As the shake flask method is based on DOC measurements, this method is not very suitable in order to evaluate the biodegradation of bio-lubricants and bio-solvents. The closed bottle method, which is based on dissolved oxygen measurements, is more suitable for these substances. An overview of the main parameters of these methods is given in Table 3, while the amount of replicates as prescribed by OECD 306 is given in Table 4.

7.1.2 International Standards

One ISO standard with regard to biodegradation of organic compounds in a marine environment is developed: ISO 16221 (2001) —Water quality—Guidance for determination of biodegradability in the marine environment. This standard is based on OECD 306, but a few modifications are made with regard to the measurement techniques and the inoculum. The measurement techniques are based on established aerobic freshwater tests. The main parameters of the test method are given in Table 5. The amount of replicates is in function of the used measurement technique

Table 3
Overview of the main parameters as described in the shake flask method and the closed bottle method (OECD 306)

Parameter	Shake flask method	Closed bottle method
Suitable test items	Min. solubility: 25–40 mg C/l Not volatile	Min. solubility: 2 mg/l (less soluble can also be tested) No adsorption onto glass
Inoculum	Natural seawater (after filtration) to which nutrients are added (phosphate buffer, $CaCl_2$, $MgSO_4.7H_2O$ and $FeCl_3.6H_2O$) DOC seawater< 20% DOC test mixture	Natural seawater (after filtration) to which nutrients are added (phosphate buffer, $CaCl_2$, $MgSO_4.7H_2O$ and $FeCl_3.6H_2O$)
Temperature	15–20 °C	
Reference material	Sodium benzoate, sodium acetate or aniline	
Measurement technique	DOC	Dissolved oxygen
Amount of test item	5–40 mg DOC/l	2–10 mg test substance/l
Duration	60 days Can be extended	28 days Can be extended on condition that the blank BOD values remain within the 30% limit of the O_2 in the test vessel (if this is not the case, results are not reliable due to interferences as wall growth and nitrification)
Validity	Reference substrate: comparable to results of ring test	Biodegradation reference substrate >60% (short time span) & comparable to results of ring test Blank respiration <30% O_2 test vessel

Table 4
Amount of replicates as prescribed by OECD 306

Method	Blank series	Reference series	Test series	Abiotic sterile control	Toxicity control
Shake flask method	2	1	2	1 (optionally)	1 (optionally)
Closed bottle method	>8	>8	>8	2 (optionally)	6

Table 5
Overview of the main parameters as described in ISO 16221

Parameter	Description
Inoculum	Natural seawater (after filtration) (bacterial concentration: ± 105 cells/ml) with nutrients (phosphate buffer & $FeCl_3.6H_2O$) or artificial seawater
Temperature	15–25 °C
Reference material	Sodium benzoate or aniline
Measurement technique	DOC die-away test (ISO 7827) (DOC measurements) Closed bottle test (ISO 10707) (BOD measurements) Two-phase closed bottle test (ISO 10708) (BOD measurements) CO_2 evolution test (ISO 9439) (CO_2 measurements) CO_2 headspace test (ISO 14593) (TIC measurements)
Amount of test item	5–40 mg DOC/l (ISO 7827) 2–10 mg substance/l (ISO 10707) 100 mg ThOD/l (ISO 10708) 20 mg TOC/l (ISO 9439) 20–40 mg TOC/l (ISO 14593)
Duration	60 days
Validity	Biodegradation reference material >60% (respirometric measurements) or >70% (DOC measurements) after 14 days

Table 6
Minimum amount of replicates as prescribed by ISO 16221

Method	Blank series	Reference series	Test series	Abiotic sterile control	Toxicity control
ISO 16221	2	1	2	1 (optionally)	1 (optionally)

(Table 6). For methods in which vessels have to be sacrificed for measurements more vessels are required.

At this moment ISO is developing a new method in order to determine the aerobic biodegradation of plastics sunk at the sea water/sandy sediment interface. This method will simulate a habitat found in the benthic zone where sunlight reaches the ocean floor (=photic zone = sublittoral zone). The proposed test setup consists of a solid phase (=sandy sediment) and a liquid phase (=synthetic seawater) in a closed, unstirred respirometer incubated at 20 °C–28 °C. The film sample, as a disk, is put on the sediment and covered by a coverslip. Biodegradation is measured by oxygen consumption [145].

Table 7
Overview of the ASTM standards with regard to biodegradation in aerobic marine environment

Standard	Description		
	Standard test	**Method for determining**	**Aerobic biodegradation of plastic**
D 6691-9	Materials in the marine environment by a defined microbial consortium or natural sea water inoculum		
D 6692-1	Standard test method for determining the biodegradability of radiolabeled Polymeric plastic materials in seawater		
D 7473-12	Standard test method for weight attrition of plastic materials in the marine Environment by open system aquarium incubations		

7.1.3 American Standards

An overview of the American standards with regard to biodegradation and weight attrition in a marine environment is given in Table 7. ASTM D 6691 and ASTM D 7473 are both referring to plastics, but ASTM D 6691 can be described as a Tier 1 test, while ASTM D 7473 is a Tier 2 test, closer to real-life conditions. In ASTM D 6691 the sample is cryogenically milled to increase the surface area and biodegradability (CO_2 production) is determined, while plastics are tested as such in ASTM D 7473 and weight loss is measured. As weight loss (=disintegration = physically fallen apart into smaller pieces) is measured, this standard cannot be used for demonstrating biodegradation (=complete mineralization to H_2O, CO_2 and biomass). ASTM D 6692 is designed in order to determine the degree of aerobic biodegradability of polymeric compounds utilized in plastic materials by determining the level of respiration of such radiolabeled carbon compounds to radiolabeled carbon dioxide. No specific American standards with regard to the biodegradation of lubricants or solvents are available.

The main principles of the American standard test methods D 6691 and D 6692, which determine the biodegradability of plastic materials in marine environments, are given in Table 8.

In spite of the fact that ASTM D 7473 is no standard test method with regard to biodegradability, the main principles of this standard are discussed in this chapter due to the relationship with ASTM D 6691. ASTM D 7473 is used to measure the weight loss as a function of time for non-floating plastic materials under continuous flow (open system) aquarium conditions. The conditions as simulated in this test are representative for aquatic environments near the coasts and near the bottom of a water body in absence of sunlight. Aquarium testing is considered a more realistic approach of a marine environment when compared to a closed flask test as an aquarium test allows flushing, exposure to a diverse population of microbes, removal of metabolic end products, re-supply of oxygen, exposure to anoxic conditions in sediment,

Table 8
Overview of the main parameters as described in ASTM D 6691-09 and ASTM D 6692-01

Parameter	ASTM D 6691-09	ASTM D 6692-01
Inoculum	Synthetic seawater with pre-grown population of at least ten aerobic marine micro-organisms Natural seawater with inorganic nutrients (0.5 g/l NH_4Cl & 0.1 g/l of $KH_2(PO_4)$)	Natural sea water with inorganic nutrients (0.5 g/l NH_4Cl & 0.1 g/l of $KH_2(PO_4)$) Marine sediment can be added to increase the microbial diversity
Temperature	30 ± 2 °C	Constant temperature (no specific temperature is mentioned)
Reference material	Cellulose, chitin or Kraft paper (control for activity of the inoculum) Sodium bicarbonate and sodium sulfite (control for CO_2 sensors)	Glucose or starch (uniformly labeled by 14C)
Measurement technique	Respirometer to measure the CO_2 production	Measurement of amount of radioactive polymer that had been mineralized to $14CO_2$ at various time points. Bottles are sacrificed at measurement. Before measuring the produced CO_2, the pH of the samples is brought to 2.5–3 followed by 6 h shaking. During this period the $14CO_2$ is trapped in a filter paper wick with an appropriate CO_2 trapping agent
Sample bottle	125 ml bottles	120 ml serum bottles
Amount of test item	20 mg per bottle	5–10 mg uniform 14C radiolabeled polymer per bottle (Specific activity > 0.1 μCi/mg and < 5–10 μCi/mg)
Duration	Normally 10–90 days	Several days to several weeks
Amount of replicates	Triplicate	At least 6 bottles per series
Validity	Reference > 70% biodegradation	No validation criteria

and exposure to seasonal temperature variation of incoming seawater and natural concentration of macro- and micronutrients. This test method can only be applied on materials, which achieve at least 30% mineralization in test method ASTM D 6691. If a test material does not reach 30% mineralization according to ASTM D 6691, it shall be considered non-biodegradable in the marine environment. The main parameters of test method ASTM D 7473 are given in Table 9.

Table 9
Overview of the main parameters as described in ASTM D 7473-12

Parameter	ASTM D 7473-12
Inoculum	(1) Continuous fresh supply of natural seawater (= oxygenated seawater) (2) Continuous fresh supply of natural seawater (= oxygenated seawater) & surface marine sediment (anaerobic processes can play a role for films placed on the sediment)
Temperature	Temperature of the natural seawater is recorded at zero time and at each sampling point. Seasonal temperature fluctuations and mesophilic and psychrophilic microbes will play a role
Reference material	–
Measurement technique	At selected time intervals, samples (triplicate) are removed from the aquarium box. The samples are rinsed and the weight of the rinsed samples is determined after drying to constant weight (35–40°C). The samples are also inspected visually (e.g., blackening of the undersides of the sample). Correction is made for soluble components. Weight loss is also calculated per unit area of film
Sample bottle	Plastic boxes
Amount of test item	0.5 by 0.5 in. pieces
Duration	180 days
Amount of replicates	3 replicates per weight determination/5 weight determinations per test
Validity	–

Table 10
Overview of the international standards with regard to marine aquatic toxicity

ISO standard	Description
10253 (2006)	Water quality—Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*
10710 (2010)	Water quality—growth inhibition test with the marine and the brackish water macroalga *Ceramium tenuicorne*

7.2 Toxicity

OECD has developed several guidelines for freshwater species. The major part of the fish toxicity tests was also developed toward freshwater fish species, but in OECD 210 also a marine fish species is recommended in order to execute the test (Sheep shead minnow (*Cyprinodon variegatus*)). ISO has already developed growth inhibition tests toward marine algal species (*Skeletonema costatum*, *Phaeodactylum tricornutum*, and *Ceramium tenuicorne*) (Table 10). The principles of these tests are comparable to the growth inhibition test on freshwater alga as prescribed in OECD

Table 11
Overview of the ASTM standards with regard to marine aquatic toxicity

Standard	Description
D 5660-96 (2009)	Standard test method for assessing the Microbial Detoxification of chemically contaminated water and soil using a toxicity test with Luminescent Marine Bacterium
E 724-98 (2004)	Standard guide for conducting static acute toxicity tests starting with embryos of four species of saltwater Bivalve molluscs
E 1191-03a (2008)	Standard guide for conducting life cycle toxicity tests with saltwater Mysids
E 1367-03 (2008)	Standard test method for measuring the toxicity of sediment associated contaminants with Estuarine and Marine Invertebrates
E 1463-92 (2004)	Standard guide for conducting static and flow through acute toxicity tests with Mysids from the West Coast of the United States
E 1562-00 (2006)	Standard guide for conducting acute, chronic and life cycle aquatic toxicity tests with Polychaetous Annelids
E 1563-98 (2004)	Standard guide for conducting static acute toxicity tests with Echinoid Embryos
E 1611-00 (2007)	Standard guide for conducting sediment toxicity tests with Polychaetous Annelids
E 1924-97 (2004)	Standard guide for conducting toxicity tests with Bioluminescent Dinoflagellates
E 2317-04	Standard guide for conducting renewal Microplate based life cycle toxicity tests with a Marine Meiobenthic Copepod

201, but these marine toxicity tests need to be executed in natural or synthetic seawater to which nutrients are added.

ASTM has developed a broad range of toxicity tests on different marine species (luminescent marine bacterium, saltwater bivalve molluscs, saltwater mysids, estuarine and marine invertebrates, polychaetous annelids, echinoid, bioluminescent dinoflagellates, meiobenthic copepod). An overview of the available standards is given in Table 11.

7.3 Standard Specifications

Standard specifications especially toward bio-lubricants or bio-solvents are not yet developed for a marine environment. The Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention) is the current legal instrument guiding international cooperation on the protection of the marine environment of the North-East Atlantic [146]. Representatives of 15 contracting Governments and the European Commission make up the OSPAR Commission.

Offshore chemicals that are identified by one of the following criteria shall be substituted if a less hazardous (or preferably non-hazardous) substitute is available:

– Listed in the OSPAR List of Chemicals for Priority Action.
– Substances considered by the authority to be of equivalent concern for the marine environment as the above-mentioned category.
– Inorganic combined with high toxicity.
– Persistent.
– Meet two of following criteria:
– Not readily biodegradable.
– High bioaccumulation potential.
– High toxicity.

The OSPAR guidelines for environmental compliance require component level testing of chemicals released to the marine environment for biodegradation, bioaccumulation, and toxicity. These standards are considered to be the most appropriate for measuring the overall impact of a substance in the marine environment [147–149]. Following criteria need to be evaluated for each component:

– Persistence (biodegradation in seawater according to OECD 306).
– Bioaccumulation (Kow).
– Marine toxicity.
– Growth inhibition test using the marine alga *Skeletonema costatum* [150].
– Acute toxicity test using the marine copepod *Acartia tonsa* [151].
– A sediment bioassay using an amphipod *Corophium* sp. [152].
– A fish acute toxicity test (recommendation: turbot juvenile) [153].

ASTM D 7081 is a standard specification, which encompasses criteria (including disintegration, biodegradation, and environmental impacts with regard to aquatic toxicity, metals, and other toxic substances) for non-floating plastics that are designed to be biodegradable under the marine environmental conditions of aerobic marine waters or anaerobic marine sediments [154]. This standard specification is intended to establish the requirements for labeling materials and products, as—biodegradable in marine waters and sediments or marine disposable. From the literature review on the biodegradation test methods in a marine aerobic environment, it can be concluded that there exist considerably

less biodegradation test methods when compared to a freshwater environment. ISO and ASTM are already more progressive as more guidelines toward marine organisms were developed. The guidance documents toward the sample preparation and the interpretation of the results of toxicity tests for difficult substances (OECD), poorly water soluble substances (ISO 14442), and lubricants (ASTM D 6081) should be taken into account.

8 Conclusion

The paradigm shift must be introduced gradually. Accepting new methods is not straightforward since they will never replace a traditional one directly; most of the time, a new method will refine the strategy, which includes the abandoning of earlier components, and in the future this may lead to a reduced need for animal data. The paradigm change in toxicity testing presents a number of opportunities for improved regulatory outcomes. The efficiency gains in terms of both time and money would improve animal testing expenses in both the public and the private sector. The focus on pathways, mechanisms, and modes of action and the use of more realistic doses could reduce some instances of excessive reaction in risk assumptions and produce more accurately targeted regulation. The biodegradation rate can be determined based on the measurement of dissolved organic carbon, dissolved oxygen, CO_2 production, oxygen consumption, or inorganic carbon.

However, several improvements are required in order to enhance the reproducibility of the results and the credibility of the testing methods. The literature review on the specifications revealed that currently no clear specifications for biodegradation in fresh water exist on European and American level. Therefore, there is an urgent need as standardization can play an important role in the uptake of products such as bio-based lubricants and bio-based solvents and consequently can help to increase market transparency by providing reference methods and criteria. There is the need for modernized OECD biodegradation test guidelines. However, the modernization of OECD guidelines may create several major challenges, including the costs of approval and application of new guidelines. The chemical industry and regulators may also have to face challenges regarding the impact of novel approaches and modified OECD tests on the future test results, especially their interpretation and overall chemical risk assessment.

Overview of new OECD guidelines would also require standardization of test systems and methods among all research laboratories that perform the biodegradability testing, to assure the same standard of quality of results, and to enable comparison between biodegradation studies conducted by different laboratories. However, there is the need for solutions that address the main issues

regarding current biodegradability testing, and application of advanced microbial ecology methods offers an opportunity to improve present OECD biodegradation test guidelines. With regard to marine environmental safety, it can be concluded that less tests were developed when compared to the freshwater environment especially on OECD level. ISO and ASTM are already more progressive as further guidelines toward marine organisms were settled.

9 Future Prospects

In my view, further simplifications of information and information flows are necessary in order to manage chemicals in the near future. The review of the marine biodegradation test methods also revealed a few items, which should be further investigated in order to optimize the test methods. Among others, the inoculum, the addition of nutrients, the difference between conditions in different parts of the sea should be further investigated.

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