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Influence of microbial adaption and supplementation of nutrients on the biodegradation of ionic liquids in sewage sludge treatment processes

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ABSTRACT

As ionic liquids are winning more attention from industry as a replacement of more hazardous chemicals, some of their structures have the potential to become persistent pollutants due to high stability towards abiotic and biotic degradation processes. Therefore it is important to determine the hazard associated with the presence of ILs in the environment, for example biodegradation under real conditions. Standard biodegradation testing procedures generally permit pre-conditioning of inoculum but do not allow for pre-exposition to the test substance. These are usually conducted in a mineral medium which does not provide additional organic nutrients. Though very valuable, as a point of reference, these tests do not fully represent real conditions. In *in situ* conditions, for example in wastewater treatment plants or natural soils and water bodies, the presence of readily available sources of energy and nutrients as well as the process of adaptation may often alter the fate and metabolic pathways of xenobiotics. Our results have shown that these are the opposing processes influencing the biodegradation rate of ILs in sewage sludge. The results have significant practical implications with respect to the assessment of biodegradability and environmental fate of ILs and other xenobiotics in environmental conditions and their potential remediation options.

1. Introduction

Industrial development during the last decades resulted in increased pollution of the environment by xenobiotics. Due to this, the need for understanding the impact of toxic compounds on microbial populations and the catabolic degradation pathways of xenobiotics has arisen. Thus standardized biodegradability and toxicity test were developed to allow for classification of xenobiotics according to the environmental hazard they pose. Bearing in mind the definition of xenobiotics, as man-made chemicals foreign to organisms which inhabit the environment, their biodegradation rate in natural soils and waters is in most cases much lower than that of natural compounds. Nevertheless structural similarities to biomolecules can result in relatively high biodegradation rates if enzymes of low substrate specificity are present. Factors which may influence this rate, among others, include microbial adaptation and availability of additional nutrients [1].

Ionic liquids (ILs) as a non-conventional class of novel solvents are becoming increasingly important owning to a number of desirable characteristics including negligible volatility, non-flammability, high thermal stability, low melting point, broad liquid range and controlled miscibility with organic compounds or water [2–5]. The negligible volatility limits their impact on air quality, but their release to the environment may affect soil and water. Moreover, some IL structures have the potential to become persistent pollutants due to their high stability towards abiotic and biotic degradation processes. Therefore it is important to determine the hazard associated with the presence of ILs in the environment.

Adaptation is defined as a change in the microbial community that leads to an increase in the biodegradation rate, or maximal biodegradable concentration of a given xenobiotic as a result of previous exposure. Examples of such adaptation processes are *e.g.* rapid degradation of p-nitrophenol by aquatic microorganisms [6] and enhanced degradation rates after elongated exposure of subsurface soil communities to m-cresol, m-aminophenol and aniline [7]. Mechanisms of adaptation usually involve processes such as genetic mutation or horizontal gene transfer, induction of specific enzymes which enhance the degradative capacity of the entire community, and population change such as selective growth of certain strains [8]. All mechanisms may take place simultaneously, or one may dominate and exact prediction of which will occur is not possible [9]. Furthermore it should be noted, that adaptation does

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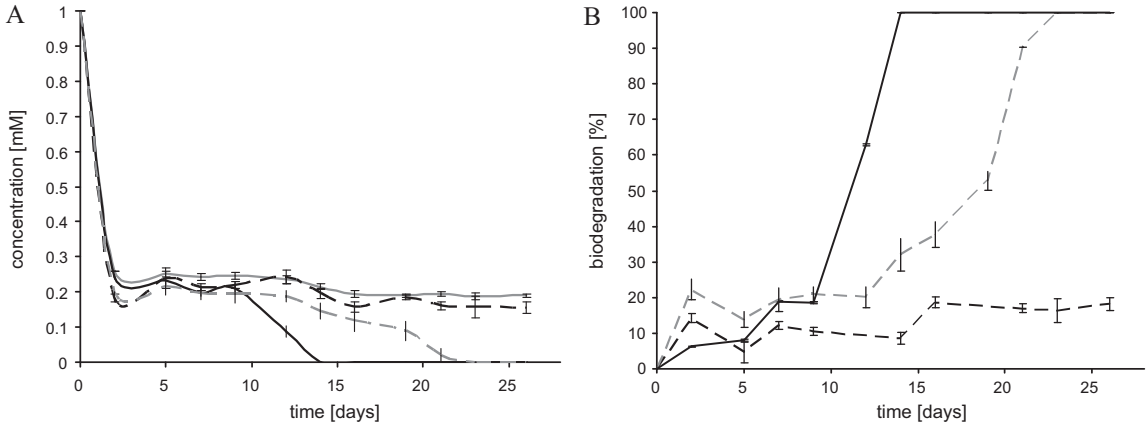

Fig. 1. (A) Biodegradation curves of [OMIM][Cl] as a sole source of carbon (—), with supplementation of glucose (- - -), with supplementation of synthetic feed (▭ ▭ ▭), sorption control (═). (B) Biodegradation (%) normalized for sorption to sewage sludge flocs of [OMIM][Cl] as a sole source of carbon (—), with supplementation of glucose (- - -), with supplementation of synthetic feed (▭ ▭ ▭).

not necessarily occur in every case. Aelion et al. did not observe any adaptation of subsurface microbial communities to chloro- and trichlorobenzene after an eight months adaptation period [7]. Similarly Nyhoim et al. did not note any increase in the biodegradation rate after pre-exposure of activated sewage sludge to aniline and pentachlorophenol [9]. The specific reason for this remains unclear though many theories exist. The most probable reasons include lack of complete enzyme systems within the population, accumulation of toxic degradation products, binding with enzymes causing inactivation or insufficient cell density of inoculum [10]. One of the few papers which discusses the adaptation of soil microorganisms to ionic liquids proposes that the electron-donor ability of the IL effect the biodegradability [11].

A number of research groups have performed biodegradation tests with alkyl substituted imidazolium cations using activated sewage sludge [12–15]. In most cases ILs were used as a sole source of organic carbon and organic nitrogen. This is especially important, because it should be remembered that in wastewater treatment plants or natural environments, other organic substrates are present, which might be preferentially degraded or co-metabolized with the primary contaminant resulting in lower biodegradation rates [16]. Romero et al. [17], discussed the biodegradability of imidazolium ILs in the presence of additional carbon source. It was found that the ILs tested were not biodegradable when D-glucose was available. However, ILs with no additional carbon were also not degraded (2–10%), which is in contrast to other research where, *e.g.* complete primary biodegradation of 1-methyl-3-octylimidazolium chloride [OMIM][Cl] was shown [12,18]. Results of Romero et al., though very interesting, should be treated with caution due to the very short duration of the test (five days) as well as lack of collaborating results in literature.

Standard biodegradation testing procedures generally permit pre-conditioning of inoculum (aeration in the presence of a mineral medium) but do not allow for pre-exposition to the test substance. The purpose of this is to provide repeatable results enabling comparison and standardization of biodegradation rates of different chemicals usually for regulatory purposes. Though very valuable, as a point of reference, these tests do not fully represent real conditions [19]. Therefore, to more accurately predict biodegradation under real conditions it is beneficial to take adaptation into account especially if biodegradation requires induction of specific metabolic pathways, *e.g.* aromatic ring break-down [6,20]. One of the few works which discuss the adaptation of microorganisms to ILs, conducted by Stolte et al., found a sixfold increase in the biodegradation rate of [OMIM][Cl] over a period of 31 days [12]. Additionally, Docherty et al. observed complete biodegradation of hexyl-methylimidazolium bromide after extending duration of the test and concluded that though IL could not be classified as readily biodegradable it is not expected to persist in the environment [20].

The aim of this paper is to describe the effect of additional substrates and pre-exposition of bacteria to IL on the rate of biodegradation, and thereby discuss the relevance of including pre-exposition in standardized tests.

2. Experimental methodology

2.1. Modified OECD 301A DOC Die-Away test – supplementation

The ionic liquid used in the test was [OMIM][Cl] provided by Merck KGA, Darmstadt, Germany. The sewage sludge (dry mass 6.5 g L^{-1}) was taken from the aeration chamber of the "Gdańsk – Wschód" municipal wastewater treatment plant, Gdańsk, Poland. Primary degradation was detected by direct determination of the substrate by HPLC – UV. Eight test flasks containing 0.5 L of sewage sludge flocs and mineral medium composed of: 8.5 mg L^{-1} KH_2PO_4, 21.75 mg L^{-1} K_2HPO_4, 22.3 mg L^{-1} $Na_2HPO_4{\cdot}2H_2O$, 1.7 mg L^{-1} NH_4Cl, 27.5 mg L^{-1} $CaCl_2$, 22.5 mg L^{-1} $MgSO_4{\cdot}7H_2O$ and 0.25 mg L^{-1} $FeCl_3$ dissolved in water were prepared as recommended by OECD procedure [21]. Subsequently, a solution of [OMIM][Cl] was added to yield the concentration of 1 mM and the amount of test solution was made up to 1 L. Each test concentration was conducted in duplicate. Two test flasks were additionally supplemented with glucose and two with synthetic sewage feed (16 g of peptone, 11 g of meat extract, 3 g of urea and 0.7 g NaCl dissolved in 1 L of water). Nutrients were added three times a week, 0.36 g and 2.5 mL, respectively. Also blank samples (without test substance) and chemically sterilized negative controls were prepared. All test vessels were aerated and analytical samples were collected in duplicate at specific time intervals. Mass loss due to evaporation was compensated at every collection interval.

2.2. Modified OECD 301A DOC Die-Away test – adaptation

The test vessels were prepared as previously described. Sewage sludge from the same source was used (dry mass 5.5 g L^{-1}). Increasing concentrations of [OMIM][Cl] (1 mM, 1.5 mM, 2 mM, 2.5 mM) were added every fortnight. The total time for the adaptation test was two months. All vessels were aerated and analytical samples were collected in duplicate at specific time intervals.

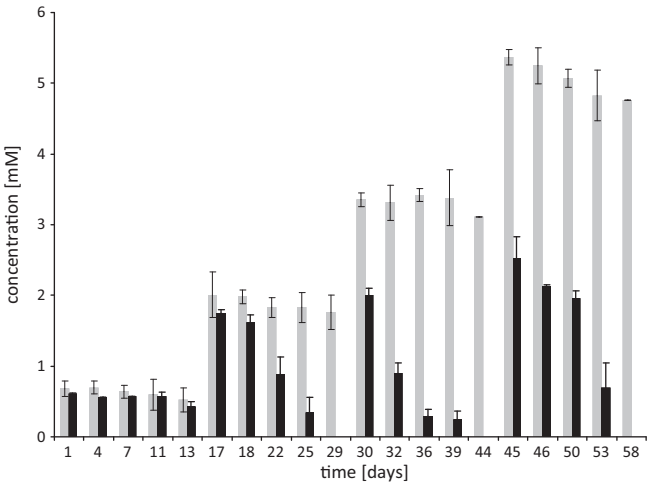

Fig. 2. Biodegradation of [OMIM][Cl] by adapted sewage sludge community (black bars) and abiotic control (grey bars).

2.3. HPLC analysis

Analytical samples were centrifuged to remove solids and a supernatant was taken for the HPLC-UV analysis. A Perkin Elmer Series 200 HPLC consisting of a chromatographic interface (Link 600) binary pump, UV/VIS detector, vacuum degasser and Rheodyne injection valve were used. For IL's cation separation C6-Phenol (Phenomenex) 150 × 4.6 mm column was used in conjunction with detection by UV adsorption at 218 nm. As a mobile phase 27% acetonitrile/water + 0.1% (v/v) trifluoroacetic acid at the flow rate of 0.8 mL min^{-1} was applied. For preparation of HPLC mobile phase HPLC – grade acetonitrile, Lab – Scan (Dublin, Ireland) and spectrophotometric grade trifluoroacetic acid (Sigma–Aldrich, Germany) were used.

2.4. Metabolites analysis

Additional analytical samples were taken from vessels containing live and chemically sterilized inocula at the end of adaptation tests. Samples were centrifuged to remove solids and the supernatant was diluted hundredfold (biotic sample) or thousandfold (abiotic sample) with a 9:1 methanol–water mixture resulting in approximately 5 μM concentration of parent compound in all samples. Subsequently samples were analyzed for the parent compound and metabolites by electrospray ionization mass spectrometry equipped with ion trap detector (Brucker-Daltonic GmbH, Germany). Mass spectra for cations were acquired in the positive ion mode in the scan range of m/z+ 50–300. The ESI source conditions were set in accordance to [22] with a capillary voltage of 3500 V, drying gas flow-rate of 5 L min^{-1}, drying gas temperature at 300 °C and nebulizer at 70 psi.

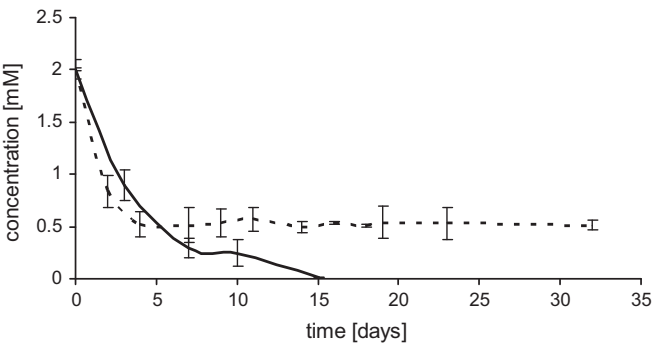

Fig. 3. Comparison of biodegradation rate of 2 mM [OMIM][Cl] conducted by adapted (solid line) and non-adapted (dashed line) activated sewage sludge community.

3. Results and discussion

3.1. Supplementation

The initial, fast decrease in [OMIM][Cl] concentration was due to sorption of [OMIM][Cl] onto flocs of activated sewage sludge (shown in Fig. 1A). The sewage sludge organic matter (especially extracellular polymeric substances) can act as a 'buffer' for the IL, initially decreasing the bioavailable concentration and thereby mitigating its toxicity. The supplementation with glucose and synthetic feed increased the time of [OMIM][Cl] primary biodegradation (Fig. 1B). It can be observed that after approximately 14 days the biodegradation of 0.2 mM [OMIM][Cl] remaining after sorption was completed only when no supplements were added. After more than 20 days biodegradation was accomplished in the sample where synthetic feed was added. In the vessel with glucose supplementation complete biodegradation was not observed within the test timeframe. [OMIM][Cl] biodegradation with synthetic feed cannot be explained using the diauxie effect [23], as the feed was added continuously for the duration of the test.

For [OMIM][Cl] as a nominal source of organic carbon and organic nitrogen complete primary biodegradation is achieved within 14 days. When synthetic feed is present the biodegradation rate is clearly reduced, even though complete primary degradation is achieved within 23 days. When supplemented with glucose, providing easily available source of organic carbon, [OMIM][Cl] is utilized in less than 20%. Therefore the presence of other nutrients in the sewage or within the environmental media in general can have a strong influence on the biodegradability of ionic liquids. Also compounds which have been classified as "readily biodegradable" might present recalcitrance towards biodegradation under real environmental conditions which has to be taken into account when evaluating their fate in the environment.

Generally, the reduced biodegradation rate of [OMIM][Cl] in the presence of glucose is consistent with the research conducted by Lewis et al. [24] where the addition of organic carbon significantly *decreased* the degradation of p-cresol. The addition of synthetic feed containing organic carbon and nitrogen decreases the rate of biodegradation (relative to non-supplemented tests). Similar results were obtained by Swindoll et al. for p-nitrophenol [25]. On the other hand, Piekarska et al. showed that the addition of other sources of organic carbon and nitrogen *increased* the efficiency of degradation of diesel oil [26]. This can be explained by the difference in the chemical structure of the primary substrates. Nitrophenol is a pure aromatic compound, biodegradation of which requires the induction of a specific metabolic pathway. Diesel oil is a mixture containing mostly linear or branched hydrocarbons, which are degraded through the β-oxidation pathway. The enzymatic systems for this pathway are relatively common in most soil and sewage microorganisms.

To summarize, addition of easily available organic carbon and nitrogen sources seems to facilitate biodegradation when the primary pollutant itself is relatively easy to degrade. It can be anticipated that xenobiotics containing structures which are commonly recognized as poorly biodegradable will not be metabolized if microorganisms can obtain carbon from other sources. This hypothesis seems to hold true when the secondary substrate is organic. Inorganic supplements were proven to facilitate biodegradation. The addition of inorganic carbon ($NaHCO_3$) and inorganic nitrogen (NH_4Cl) has previously been shown to increase the rate of biodegradation of xenobiotic compounds [9,24]. Utilization of

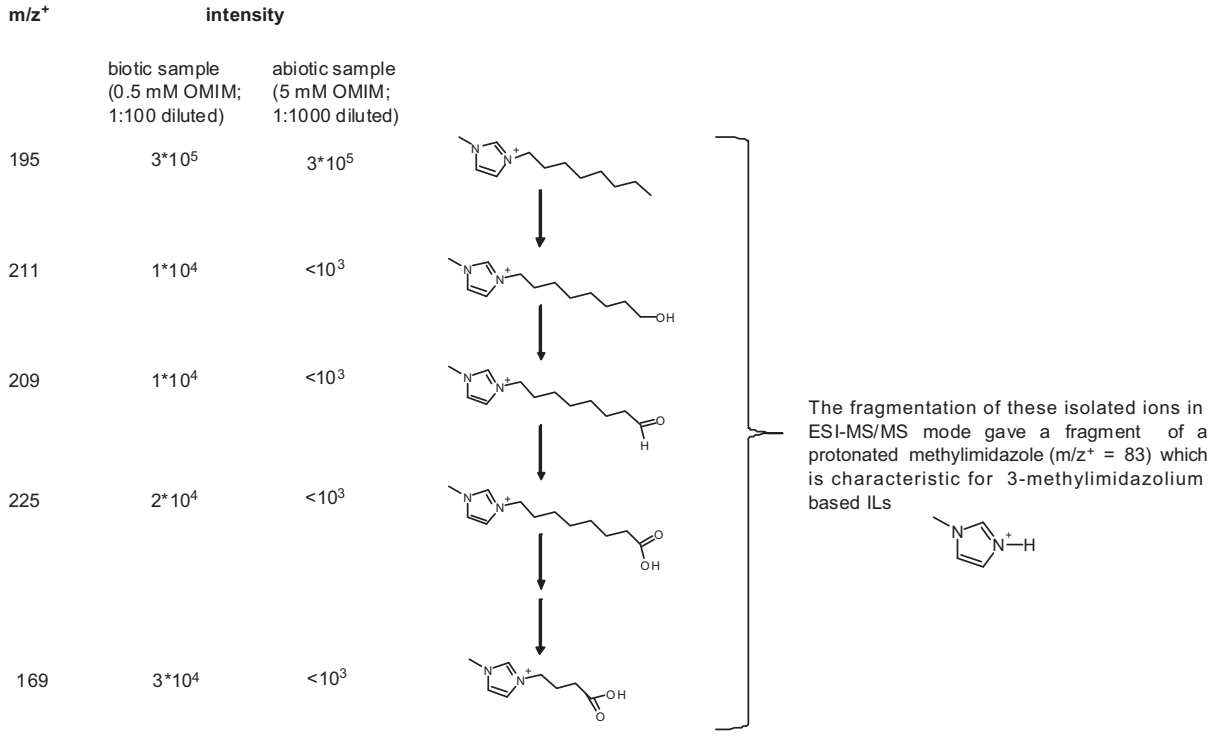

Fig. 4. Mass-to-charge ratio (positive mode), intensity of signals within the mass spectra and proposed chemical structures of degradation products.

organic carbon/nitrogen requires cleavage of these elements from the carbon skeleton before it can be assimilated by the cell, therefore it is a less energetically favorable source of these elements [27]. If both primary and secondary substrates are organic usually a similar set of reactions is needed for their break down which results in a competition for enzymes.

It should be mentioned that the concentration of [OMIM][Cl] degraded in the non-supplemented test was higher than in any previously published work [18]. Thus it can be assumed that in wastewater treatment plant operating conditions this substance might be treatable as defined by OECD standards. The high level of sorption of [OMIM][Cl] will also allow for enhanced removal.

3.2. Microbial adaptation

Municipal sewage sludge was exposed to gradually increasing levels of [OMIM][Cl] added in fortnightly intervals. Progressive accumulation of the xenobiotic can be observed in abiotic control (Fig. 2). Theoretical total concentration anticipated in all samples at the end of the test, excluding biodegradation and sorption processes, is 7 mM. Concentrations measured in aqueous phase of test media in biotic and abiotic control are presented in Fig. 2.

In non-sterilized sample sewage sludge was able to adapt to the IL. An initial lag phase of around two weeks was observed. The biodegradation rate for each addition increased from 1% day^{-1} to 6% day^{-1}, to 8% day^{-1} after the final addition (percentage degraded relative to the amount added for each period). The degradation data was fit using first order kinetics according to Paul and Clark [28], the corresponding rate constants where calculated to be 0.065, 0.13, 0.27, and 0.13 s^{-1}. The half-life of the contaminant in the test for each addition of IL was therefore 10.5, 5.3, 2.6, and 5.5 days. It should be noted that with each addition the concentration of IL is also raised, which might account for the lowered calculated rate constant of the last addition. The adaptation has allowed the sewage sludge to degrade concentrations of [OMIM][Cl] previously reported to be too high [18]. A small decrease in [OMIM][Cl] concentration in sorption control was observed possibly indicating that sterilizing agent did not inhibit biodegradation completely, however sufficiently to allow for distinction from sorption. Considering the fact that within the final sample (day 58, Fig. 2) a total of 7 mM OMIM was degraded and no transformation products were detected via HPLC-UV (the imidazolium core is responsible for UV absorption) it is reasonable to believe that the whole structure, including the imidazolium core, was biodegraded.

Fig. 3 shows biodegradation curves for adapted and non-adapted communities. No biodegradation of [OMIM][Cl] in concentration of 2 mM was observed for the non-adapted community. No adaption was observed in this case, probably due to toxicity of the IL at this concentration. The initial decrease in concentration is due to sorption on sewage sludge flocs, as previously shown. The adapted community, however, was able to utilize the IL. Complete primary biodegradation was achieved within 15 days.

The results from ESI-MS analysis have been used to identify biological transformation products. Therefore, the biotic and abiotic samples after the course of biodegradation (53 days, Fig. 2) have been used for a qualitative analysis. The ESI-MS analysis indicates that several biological transformation products were formed such as compounds with hydroxylated or carboxylated side chains (Fig. 4, for mass spectra see supplementary data). The identified metabolites correspond to the recently proposed degradation pathway of the OMIM cation [12] starting with an ω-oxidation (introduction of a terminal hydroxyl group) and a subsequently degradation of the alkyl side chain via β-oxidation. Also in the abiotic sample some biodegradation products could be identified with very low peak intensities (not present in the OMIM standard) confirming that inhibition of inoculum was not complete.

4. Conclusions

In *in situ* conditions, for example in wastewater treatment plants or natural soils and water bodies, the presence of readily available sources of energy and nutrients (*e.g.* sugars, fats or proteins) may

often alter the fate and metabolic pathways of xenobiotics. Our results have shown that there are two opposing processes influencing the biodegradation rate of ILs in sewage sludge. The results have significant practical implications with respect to the assessment of biodegradability and environmental fate of ILs and other xenobiotics in environmental conditions and their potential remediation options. In the activated sewage sludge process, the excess sewage is sequentially removed, however adapted communities of microorganisms are expected to remain in the reactor. The significance of adsorption on sewage sludge flocs as a way of xenobiotic removal during the wastewater treatment cannot be overstated. The typical time of hydraulic retention of sewage in the bioreactor is approximately one day and is too short for the biodegradation of most xenobiotics to occur. However these contaminants usually do not persist in the wastewater treatment plant effluents, and therefore the removal by physical adsorption on flocs which are subsequently removed is of a great importance [19].

In present case, adaptation promotes the removal of ILs. It should be noted that not only is it increasing the rate of biodegradation, but also increasing the maximum biodegradable concentration. It was shown hereby that through the process of adaptation a nearly 30-fold increase of the biodegradation rate can be obtained. Additionally concentrations of ILs previously reported to be too high to be metabolized by non-adapted communities were degraded. Moreover complete degradation of imidazolium ring was observed. It can be anticipated that during wastewater treatment of IL in an activated sewage sludge process similar phenomena will occur.

On the contrary, the supplementation with organic carbon or nitrogen diminished the rate of biodegradation. The microbial community preferentially utilized secondary supplements and ILs persisted. This contradicts the description of ready biodegradability by OECD which states that "*it is assumed that such* [readily biodegradable] *compounds will rapidly and completely biodegrade in aquatic environments under aerobic conditions*". It was shown that supplying both organic carbon and nitrogen has a less detrimental effect than supplying only carbon. In the first case the observed lag phase was long but was eventually followed by degradation whereas in the latter complete inhibition of biodegradation was observed.

It should be noted that the biodegradation of every xenobiotic is determined with standardized laboratory procedures. The results obtained from these tests provide information allowing for comparison and classification of chemicals according to their biodegradability. However the results of these tests cannot be easily extrapolated to environmental conditions. According to OECD guidelines if a substance reaches thresholds of ready biodegradability in 301 series of tests it should be easily and rapidly degraded in the environment. We have shown hereby that this is not always the case.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.08.053.

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