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Evaluation of biodegradation of nonylphenol ethoxylate and lignin by combining toxicity assessment and chemical characterization

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ABSTRACT

The aerobic biodegradation of commercial nonylphenol ethoxylate (NPE) mixture and alkali lignin was studied using the OECD headspace test accompanied by the simultaneous measurement of ecotoxicity directly from the biodegradation liquors and by the follow-up of the chemical composition of the studied chemicals. NPE degradation was dependent on the inoculum source: approximately 40% of NPE was mineralized into CO_2 during the 4-week experiment when inoculum from Helsinki City wastewater treatment plant (WWTP) was used, and only 12% was mineralized when inoculum from Jyväskylä City WWTP was used. Chemical analyses revealed a shift in the ethoxylate chain length from longer to shorter soon after the beginning of the NPE biodegradation tests. At the same time also toxicity (reverse electron transport assay, RET) and estrogenic activity (human estrogen receptor yeast) measured directly from the biodegradation liquors decreased. In case of alkali lignin, approximately 11% was mineralized in the test and chemical analysis showed in maximum a 30% decrease in lignin concentration. Toxicity of lignin biodegradation liquors started to decrease in the beginning of the test, but became more toxic towards the end of the test again. Especially RET assay proved to be sensitive enough for measuring toxicity changes directly from biodegradation liquors, although a concentrating treatment of the liquors is recommended for a more detailed characterization and identification of toxic metabolites.

1. Introduction

Biodegradation tests on substances, or chemical preparations, supplemented with separate analyses of the biological effects of the identified degradation intermediates have been used in higher tier risk assessments for quite a long time. Recently, toxicity testing of biodegradation intermediates directly from biodegradation test liquors has been suggested as one alternative method to be used in the complementary risk assessment required for surfactants, which are considered likely to produce recalcitrant metabolites (EC, 2004, 2005). The methodology presented in the EU Commission Recommendation (EC, 2005) is quite time consuming, as the metabolites are enriched prior to toxicity testing by adding periodically new parent compound into the test system.

An extensive combination of chemical and biological methods to standard biodegradation tests could give valuable information also on other organic compounds than surfactants. However, it seems that scientific studies that link ecotoxicological assays directly to standardized aquatic biodegradation tests are, in fact, quite scarce. Detailed knowledge of the biodegradation process, its products and biological effects is important, for example, for the operation of wastewater treatment plants (WWTPs). They receive huge loads of organic compounds, of both anthropogenic and natural origin, which (or their degradation products) end up within effluents into the receiving waters or within sludge onto soils. Alkylphenol ethoxylates (APEs) and lignin are examples of such compounds.

APEs, nonylphenol ethoxylates (NPEs) being the most abundant, are manmade surface active compounds that contain hydrophilic ethoxy groups attached to the lipophilic alkylphenol unit. NPEs are widely used around the world, for example, as detergents in industry and households, though their use today is restricted at least within the EU (EC, 2003). Despite this, they are still likely to appear in many European wastewater streams, as is the case in Finland (unpublished monitoring data). Biodegradation of NPEs occurs through aerobic and anaerobic processes. Their degradation intermediates are considered more harmful than the parent chemicals and their harmful property has been linked especially to the endocrine disrupting potential of nonylphenol (NP) (e.g. Jobling and Sumpter, 1993; White et al., 1994; Routledge and Sumpter, 1996; Isidori et al., 2006).

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Lignin, on the other hand, is an abundant natural biopolymer being one of the key components in wood. Industrial pulping processes aim at removing lignin in order to obtain colorless good quality pulp. Alkaline cooking processes result in the increase of the water soluble lignin fragments in the pulping liquor and typically phenolic groups are liberated (Alén, 2000). Also the subsequent bleaching steps modify the chemical composition of lignin. Parts of these modified lignin compounds end up in wastewater streams and finally at the treatment plants. Biodegradation of lignin is very slow, and the short residence time at the treatment plant does not promote degradation of lignin either. Wastewaters that contain lignin have a strong color and may have harmful biological effects (Higashi et al., 1992; Pillai et al., 1997; Pessala et al., 2004).

In our study we combined biodegradation studies with bioassays and chemical analyses, the specific aims being: (1) to examine the aerobic biodegradation of two organic preparations (NPE mixture and alkali lignin) in 28-d tests; (2) to determine the suitability of the selected bioassays to direct effect assessment from biodegradation test liquors; (3) to follow-up changes in the chemical composition of the studied chemicals and their possible influences on toxicity and other biological responses.

2. Experimental

2.1. Chemicals

The NPE mixture was Synperonic NP10 from Sigma–Aldrich/Fluka. Alkali lignin was obtained from Sigma–Aldrich.

2.2. Biodegradation

Three 28-d headspace tests (OECD, 2006) were carried out; two with Synperonic NP10 (tests No. 1 and 2) and one with alkali lignin (test No. 3). Activated sludge, which was aerated in laboratory for several days in order to remove organic carbon and after that diluted to 4 mgSS L^{-1}, was used as test inoculum. In test No. 1 the inoculum originated from Helsinki City WWTP (domestic and industrial wastewaters), in test No. 2 from Jyväskylä City WWTP (domestic and industrial wastewaters) and in test No. 3 from a pulp and paper mill WWTP (industrial wastewaters). The test compound concentration was 20 mgC L^{-1} (28.0 mg Synperonic L^{-1} and 38.2 mg lignin L^{-1}). Test bottles (test volume 83 mL) were incubated in the dark at 20 °C on a shaker. Altogether 5 or 6 replicate bottles were prepared for each sampling date, of which three were used for the mineralization measurements and the rest were used for the bioassays. Mineralization was monitored at least every 7 d by measuring the inorganic carbon levels (CO_2 in tests No. 1 and 3, and CO_3^{2-} in test No. 2) of the headspace and by comparing it to theoretical values. Acidification or alkalinization of the test bottles, which was needed for the CO_2 and CO_3^{2-} measurements, hindered bioassays on these same bottles. CO_2 was measured with Easy Quant (EQ 90) Universal carbon analyzer and CO_3^{2-} with Shimadzu (TOC 5000A) carbon analyzer. Aniline was used as the reference chemical. Sterile controls (test medium, inoculum and test compound) were obtained by autoclaving a few test bottles, and chemical controls without the inoculum were analyzed three times during the 28-d incubation period (see Table 1). These controls were used to assure that there was no background production of CO_2. The bioassay bottles from tests No. 1 and 3 were ultrasonicated for 15 min prior to bioassays, in order to get the particles dissolved and the samples more homogenous.

2.3. Bioassays

Adenosine triphosphate (ATP) content of the bioassay bottles (tests No. 1 and 3) was measured using the ATP kit from Aboatox Oy, Finland. ATP analysis can be used as a measure of the living biomass, as the intracellular level of ATP is similar in all living cells being rapidly degraded when the cell dies. Sample volume was 100 μL and the ATP from the samples was released by adding 100 μL of ATP releasing reagent (1 min incubation). Six hundred microliters of Tris-acetate buffer (pH 7.75) and 200 μL of ATP monitoring reagent were added and the luminescence of the sample was measured using Bio-Orbit 1257 luminometer. Finally 10 μL of internal standard (1000 pmol ATP mL^{-1}) was added, luminescence measured and the ATP content (pmol mL^{-1}) of the samples was calculated.

Reverse electron transport (RET) enzyme inhibition test was performed to all bioassay bottles. RET test is based on the enzymatic electron transport on the beef heart mitochondrial inner membrane (Knobeloch et al., 1994; Read et al., 1998). Two parallel determinations were performed on all test samples, the highest test concentrations being 78%. In case of concentrated samples, also solvent control (3.5% DMSO) was used. Results were calculated as averages from the two parallel measurements, which were carried out alternatively with iEMS Ascent microplate reader (Labsystems, Finland) or Victor^3 1420 multilabel counter (Perkin–Elmer).

Estrogenic activity of the samples was studied using an estrogen receptor (ERα) yeast assay in test No. 1. Detailed information on the construction and cultivation of the recombinant yeast can be found in the literature (Leskinen et al., 2003, 2005). The test is based on two *Saccharomyces cerevisiae* yeast strains: one including human ER inducible *luc* reporter genes, the other producing light constitutively (Contr). The test was carried out on white 96-well microplates containing 50 μL of yeast suspension and 50 μL of

Table 1
RET test results from the three different biodegradation tests presented as EC20 values (percentages of the original biodegradation liquor). Results are expressed as the mean of replicate bottles with corresponding standard deviations (in brackets).

Day	Test No. 1			Test No. 2				Test No. 3		
	Test	Blank	Chemical control	Test	Blank	Test (10 × conc.)	Blank (10 × conc.)	Test	Blank	Chemical control
0	9.2 (0.3)	35.5 (2.5)	8.0 (0.6)	27.4 (2.0)	>78	3.9 (0.5)	>78	17.6 (0.4)	>78	15.5 (0.6)
1	na	na	na	38.7 (7.0)	>78	<2.5	>78	na	na	na
3	na	na	na	>78	>78	40.5 (10.9)	>78	na	na	na
7	37.6 (5.7)	44.7 (5.3)	na	>78	>78	>78	>78	32.2 (5.1)	61.7 (9.1)	na
14	35.5 (0.9)	42.2 (1.0)	10.6 (0.03)	na	na	na	na	29.4 (2.4)	62.9 (2.8)	21.6 (1.7)
21	39.4 (1.9)	50.7 (0.9)	na	na	na	na	na	26.9 (1.9)	71.8 (1.8)	na
28	36.6 (5.1)	43.5 (3.3)	11.1 (0.04)	na	na	na	na	21.0 (2.7)	63.0 (0.5)	20.8 (0.9)

Test = bottles with test medium, inoculum and test compound.
Blank = bottles with test medium and inoculum.
Chemical control = bottles with test medium and test compound.
na = not analyzed.