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Influence of nonylphenol on the microbial community of lake sediments in microcosms

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Abstract

In this study the impact of nonylphenol, an estrogenic degradation product of alkylphenol polyethoxylates, on the microbial community structure in contaminated sediments of aquatic microcosms using in situ hybridization with fluorescently labeled oligonucleotides probes was investigated. A positive correlation between nonylphenol concentration and cell numbers of bacteria and microfungi as well as an increase in the numbers of active bacteria was found. However, the ratio between total microorganisms and active bacteria remained unchanged. A large fraction of the cells could be identified using group specific oligonucleotide probes. A slight change in the composition of the microbial community structure was observed, with Gram-positive bacteria with high DNA G + C-content becoming more abundant at higher concentrations of nonylphenol. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Nonylphenol; Exotoxicology; Microbial community structure; In situ hybridization

1. Introduction

The pollution of aquatic ecosystems by non-ionic surfactants of the alkylphenol polyethoxy-

late type and their degradation products have received increasing attention over the last decade due to their estrogenic effects on wildlife (e.g. Jobling et al., 1996; Baldwin et al., 1997). However, although microbes play an important role in nutrient cycling in all kinds of environment, little is known about the impact of these chemicals on bacteria and fungi in ecosystems and in particular on the structure of microbial communities.

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Most attempts to describe aquatic sediment microbial communities are based on cultivation and were therefore subject to restrictions and biases leading to a distorted representation of the true community composition (Amann et al., 1995). Molecular techniques, however, have greatly increased our knowledge of aquatic and microbial diversity (Schulze, 1999; Huber, 1997) suggesting the presence of hitherto uncultured microbes. Techniques such as reassociation analysis of DNA (Torsvik et al., 1996), denaturing gradient gel electrophoresis (Teske et al., 1996), and restriction fragment length polymorphism (Moyer et al., 1994) have yielded insight into bacterial diversity and community composition. Also fluorescence in situ hybridization (FISH) has been applied successfully to analyze bacterial communities in environmental samples such as activated sludge, multispecies bio films, trickling filters, soils and sediments by using fluorescently labeled oligonucleotide probes targeting to the 16S or 23S rRNA (Amann et al., 1995; Snaird et al., 1997; Zarda et al., 1997; Bruns and Berthe-Corti, 1998; Stoffels et al., 1998) which has made an cultivation independent identification of the microbes on different phylogenetic levels possible.

The aim of this study was to investigate possible correlations between bacterial community structure and nonylphenol concentration in artificially polluted sediments of microcosms using fluorescent in situ hybridization. All data shown in this paper are focused on the late application period (day 36).

2. Materials and methods

2.1. Microcosms

Containers (\varnothing 80 cm, height 60 cm) made from stainless steel were filled with 10 cm of lake sediment and 230 l of water from Lake Ammersee (Bavaria, Germany) each and installed in an artificial outdoor pond to maintain natural temperature. Technical nonylphenol (Sigma, Aldrich) was applied using a controlled release technique (Pfister et al., 1999) to maintain seven concentra-

tion levels (9, 27, 41, 50, 83, 92, 112 $\mu\text{g}/\text{l}$) (N1–N7) in the water column. The *application period* lasted 45 days, followed by a period without application of nonylphenol (*post-application period*) of 45 days. Four microcosms with no addition of nonylphenol served as controls.

The redox-potential in the sediments of each microcosm was measured weekly using a micro-electrode (WTW, Germany) in the surface of the sediment (0–1 cm) and in a deeper layer (1–4 cm) separately.

During application and post-application sediment cores were taken for chemical as well as for microbiological analysis from each microcosm in three replicates by using a transparent tube (\varnothing 26 mm, length 60 cm). For further analysis the upper 1-cm was cut and mixed to provide homogenous samples.

2.2. Determination of technical nonylphenol (NP) in the sediment samples

Sediment aliquots of 2–10 g were taken for HPLC-analysis and 0.5–1 g for moisture determination. For HPLC-analysis samples were suspended in 200 ml of water (HPLC grade) and 50 μl of a 10 $\text{ng}/\mu\text{l}$ solution of *n*-nonylphenol in acetonitrile were added as internal standard. The suspension was acidified to pH 2 using 2.5 N HCl.

The extraction lasted 3 h using a modified steam-distillation/solvent-extraction apparatus and iso-hexane as solvent as described by Veith and Kiwus (1977). After removal of the solvent, the residue was filled up with acetonitrile to a total volume of 1 ml.

The extract was analyzed using a VARIAN HPLC system (250 \times 3 mm column, Purospher RP18e, 5 μm , Merck, Darmstadt, Germany) under fluorescence-detection (230-nm excitation, 310-nm emission). As further conditions an eluent flow rate 0.7 ml/min, a column temperature 20°C, and a gradient from acetonitrile/water 70/30 to 100% acetonitrile in 7 min, hold at this composition for 5.5 min, was used. Under these conditions the components (mainly isomers) of technical nonylphenol (tNP) were eluted almost in one peak and with good separation from the

internal standard peak. Quantitative results were obtained on the basis of the grouped peak area of TNP with the internal standard method.

2.3. Microbiological analysis

For determination of total cell numbers and in situ hybridization, sediment samples were treated with a fixative for at least 4 h (4% paraformaldehyde PBS) (Amann et al., 1990). After washing in PBS, the fixed sediment was resuspended and finally stored in a 1:1 mixture of PBS and 96% ethanol at -20°C . For cell disaggregation, samples were diluted in 0.1% sodium–pyrophosphate buffer and 0.1% iminodiacetic acid and treated by mild sonification for 30 s.

Total cell counts were determined by membrane filtration of the sediment and staining with 4',6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980).

All specification and target position of the used probes for in situ hybridization are given in Table 1. Oligonucleotide probes labeled with fluorescent dyes CY3 were purchased from Interactiva (Germany). Fixed samples were immobilized on glass slides by air drying. Cells were further fixed and dehydrated by immersing the slides in 50, 80

and 95% ethanol (Amann et al., 1995). In situ hybridizations were performed as described by Snaidr et al. (1997). Slides were examined with an Axiovert microscope (Zeiss, Germany).

3. Results and discussion

3.1. Characterization of the sediments

The redox-potential of the sediment surface layers in microcosms showed a characteristic picture which is mainly based on a seasonal run with a tendency to lower values oxygen concentrations in the autumn period (Fig. 1). An additional influence of the nonylphenol could not be observed. Similar data, but with lower values ranging from 0.1 to -0.3 V were observed in the deeper layers of the sediment samples (data not shown). These data clearly indicate that the used experimental setup is very close to natural lake conditions as the measured values are nearly identical to those found in lake Ammersee over a years period (0.6–0.3 V) (data not shown).

Also dissolved organic carbon and nitrogen were analyzed in the sediment samples during the experimental period. The obtained data showed a

Table 1
Characterization of the used oligonucleotide probes

Probe	Specificity	Target position (rRNA) ^a	Reference
EUB 338	Bacteria	16S, 338–355	Amann et al. (1990)
ALF 1b	Alpha subclass of <i>Proteobacteria</i>	16S, 19–35	Manz et al. (1992)
BET 42a	Beta subclass of <i>Proteobacteria</i>	23S, 1027–1043	Manz et al. (1992)
GAM 42a	Gamma subclass of <i>Proteobacteria</i>	23S, 1027–1043	Manz et al. (1992)
CF 319a + b	<i>Cytophaga-Flexibacter</i> -subphylum	16S, 319–336	Manz et al. (1996)
HGC 69a	High G + C Gram-positive bacteria	23S, 1901–1918	Roller et al. (1994)
LGC 345a + b + c	Low G + C Gram-positive bacteria	16S, 354–371	Meier (1997)

^a *E. coli* numbering according to Brosius et al. (1981).

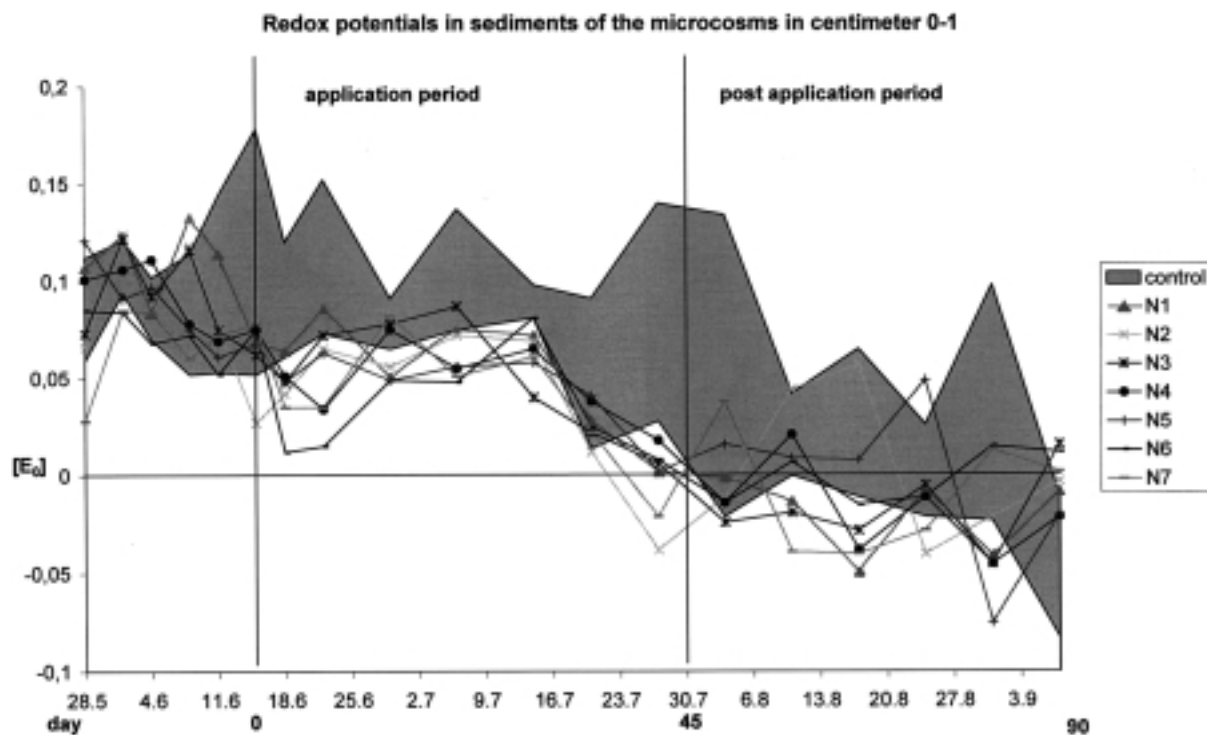


Fig. 1. Redox potential in the surface layer of the sediments from all microcosms.

typical seasonal run in all microcosms and did not differ significantly from those found in lake Ammersee (data not shown).

3.2. Determination of the nonylphenol concentration

During the application period, on day 36 concentrations in the sediments were 162, 322, 686, 697, 532, 1088 and 2024 $\mu\text{g}/\text{kg}$ dry weight (N1–N7). The results clearly show a close correlation between the amount of applied tNP and the NP concentration in the sediment. Nevertheless it has to be considered that these values present only one time point (day 36) and might be therefore not representative for the time course of the tNP concentrations and the total burden of each microcosm.

3.3. Total and active cell counts

The same day total cell counts (DAPI-staining)

and the number of active bacterial cells (EUB338) were determined (Fig. 2). Total cell numbers (bacteria and microfungi) and counts of active cells were significantly higher at the two microcosms with the highest NP concentrations in the sediments (Dunnett test and Student–Newman–Keuls method). In all microcosms more than 50% of the cells visualized by DAPI staining were also detected by the probe EUB 338, which is specific for all *Eubacteria*. Similar yields were obtained for wadden sea sediments (Llobert-Brossa et al., 1998) and activated sludge (Snaird et al., 1997). The ratio of EUB/DAPI did not change significantly with different amounts of NP.

These results (DAPI-staining and oligonucleotide probing) clearly indicate that there has been no toxic effect of NP on microorganisms. In contrast, a stimulating effect on the bacteria could be observed with increased NP concentrations. Possibly NP was used as an additional substrate by the sediment microorganisms or, alternatively, sedimentation of dead zoo- and phytoplankton,

affected by the contaminant, provided additional food resources for the microbes.

3.4. Abundance of major bacterial groups

The microbial community composition of three microcosms (control, N1 and N7) was further investigated by FISH. Using a set of six probes (ALF 1b, BET 42a, GAM 42a, CF 319a + b, HGC 69a, LGC 345a + b + c) for major phyla within the domain bacteria it was possible to identify 67% (control microcosm), 88% (N1) and 87% (N7) of the total cell counts. The in situ hybridizations of the sediment samples with probes for the major phyla revealed no significant differences in the group specific cell counts (Fig. 3a–c). In all three samples bacteria belonging to the α -, β -, and χ -subclasses of *Proteobacteria* were most abundant. Interestingly, in the untreated microcosms only <1% of the cells visualized by DAPI-staining could be hybridized with the probe

HGC 69a (Fig. 3a), that is specific for Gram-positive bacteria with a high DNA G + C content. In the dosed microcosms (N1 and N7) 9% of the DAPI-stained cells could be detected with this probe (Fig. 3b,c). In the sediment of the control microcosm in contrast higher numbers of bacteria belonging to the *Cytophaga-Flavobacterium* cluster (11% of DAPI counts) were detected as compared to the treated microcosms sediments (N1: 3% and N7: 1%). Llobert-Brossa et al. (1998) identified this phylogenetic group as the most abundant one in wadden sea sediments. This was an interesting result, because members of the *Cytophaga-Flavobacterium* cluster are mainly aerobic, Gram-negative bacteria specialized for the degradation of complex macromolecules.

Further experiments are required to show that the observed shifts in the composition of the bacterial community structure are due to the exposition with NP.

A more detailed investigation regarding a pos-

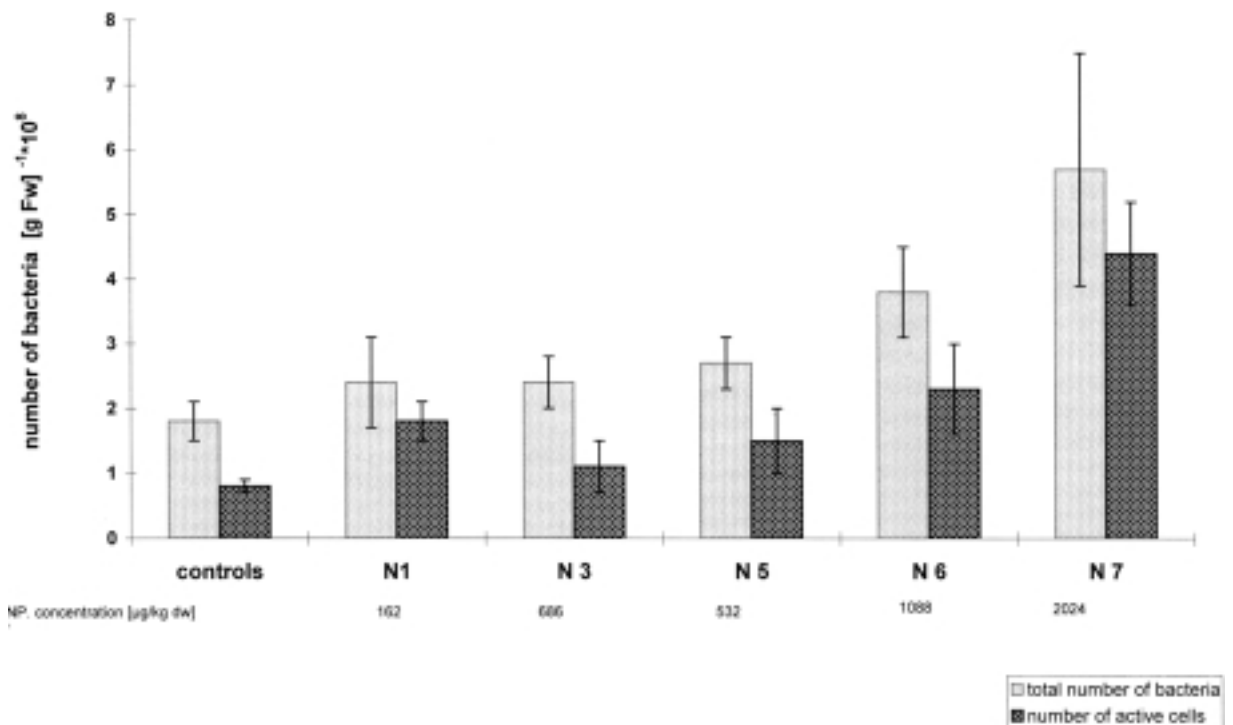


Fig. 2. Abundance of the total number of bacteria (DAPI counts) and active number of cells (EUB counts) in microcosms at day 36 after the start of application ($P < 0.05$, error bars are 95% confidence intervals).

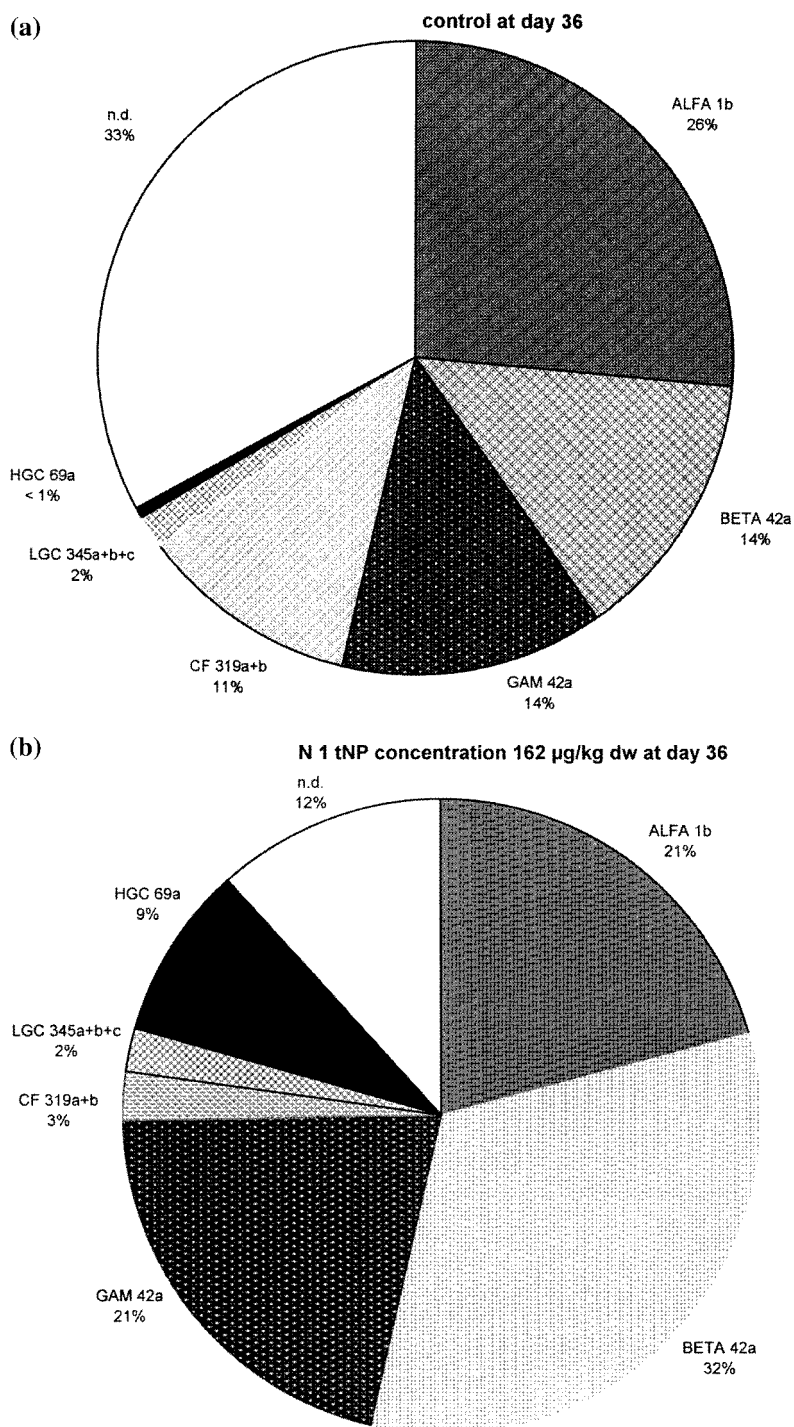


Fig. 3. Distribution of different bacterial groups (in situ hybridization) in the microcosms control N1, N7, 36 days after the start of application.

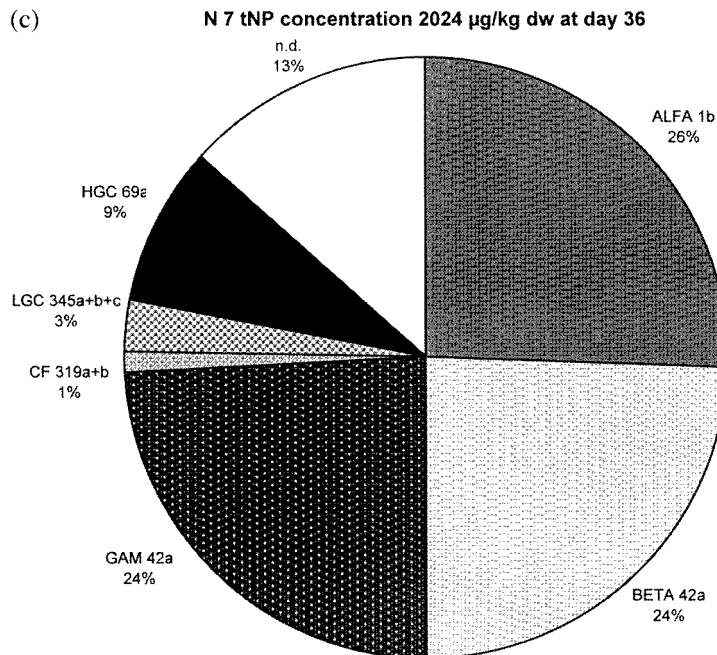


Fig. 3. (Continued).

sible impact of NP on the microbial community structure will identify bacteria to genus or species level by dot blot hybridization or denaturing gradient gel electrophoresis (DGGE) after amplification of special sequences of 16S rRNA.

The latter method was also used to investigate the composition of the natural microbial community of lake Ammersee sediment to those of the microcosms. At least in the early phase of the experiment (application period) no significant changes in the microbial community structure were observed between lake Ammersee sediment and the sediment of the control microcosms (data not shown).

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