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**Influence of trenbolone on the structural and functional diversity of microbial
communities from a lake sediment**

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“O mestre fazeis com que eu procure mais....”

Para o meu querido Dommi

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II. List of Abbreviation

AMC	aminomethyl-coumarine
bp	base pairs
BrdU	5-bromodeoxyuridine
BSA	bovine serum albumin
cDNA	complementary DNA
DAPI	4'-6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
dH₂O	distilled water
DNA	deoxyribose nucleic acid
DNase	deoxyribonuclease
e.g.	for example
EDCs	endocrine-disrupting chemicals
EDTA	ethylene diamine tetra acetic acid
et al.	et alteri
FISH	fluorescence in situ hybridization
g	gram
h	hours
kg	kilogram
L	liter
LDPE	low-density polyethylene
M	molar
min	minute
mRNA	messenger RNA
MUF	methylumbellifene
NH₄⁺	ammonia
NO₃⁻	nitrate
°C	degree centigrade
PCR	polymerase chain reaction
PEG	polyethylene glycol
pmol	pico moles
PO₄³⁻	phosphate
RAPD	random amplified polymorphic DNA
rDNA/RNA	ribosomal DNA /RNA
RFLP	restriction fragment polymorphism
RNA	ribose nucleic acid
RT	reverse transcription
sec	seconds
STP	sewage treatment plant
TBOH	trenbolone
V	volt
α	alpha (subgroup of the <i>Proteobacteria</i>)
β	beta (subgroup of the <i>Proteobacteria</i>)
δ	delta (subgroup of the <i>Proteobacteria</i>)
γ	gamma (subgroup of the <i>Proteobacteria</i>)

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1. Introduction

In recent years it has become evident that endocrine system of a wide range of organisms is affected by numerous chemical released into the environment. The occurrence and fate of these pollutants as well as their effects on aquatic organism were recognized as emerging issues in the environmental research.

Although effects of endocrine-disrupting chemicals (EDCs) on vertebrates have been deeply investigated, less is known about their interactions with non-target organisms, e.g. microorganisms. Microbial communities represent a key trophic level in aquatic ecosystems and carry out functions, which are essential for the ecology of sediments. They are responsible for the bulk of energy flow and nutrient cycling in those ecosystems. The degradation/mineralization of organic matter by microorganisms liberate nutrient to higher organisms and contribute to sediment diagenesis. Microbes also constitute an important source of biomass for predators. Therefore, changes on benthic microbial communities could have consequences for higher trophic levels and for the whole aquatic environment. Moreover, microbes may help to maintain the ecosystem, as they are often the main responsible for the degradation of contaminants. Therefore, proper environmental risk assessment of EDCs on aquatic ecosystems should include studies of microbial community responses.

1.1. EDCs in aquatic ecosystems

1.1.1. Origin of EDCs

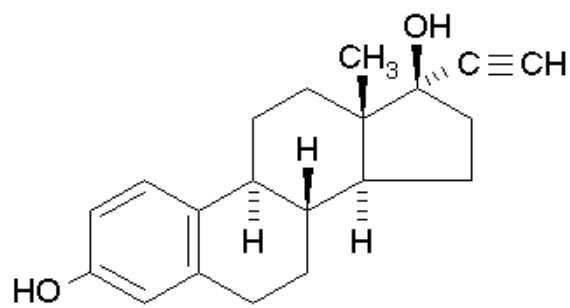
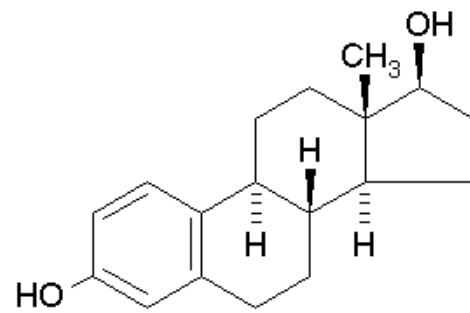
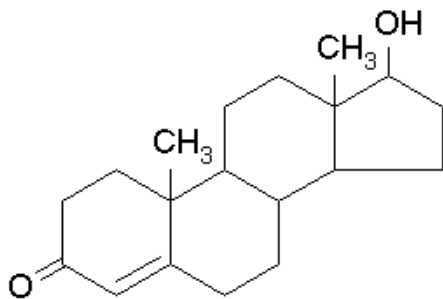
EDCs are substances that affect the endocrine system function by disrupting or modulating synthesis, secretion, binding, action or elimination of endogenous hormones in the body leading to changes on homeostasis, development, reproduction or behavior of organisms (Kavlock et al., 1996). Compounds holding endocrine-disrupting activity can be of natural or xenobiotic origin (structural formulas of commonly detected EDCs are shown in figure 1). Natural EDCs are represented mainly by natural steroid hormones, such as estradiol and testosterone, or some estrogenic substances found in plants, e.g. genistein and β -sitosterol (Spengler et al., 2001). Most of the xenobiotic EDCs originate from industrial chemicals, such as nonylphenol or bisphenol A, or are synthetic hormones (e.g. 17 β ethinylestradiol) used for

pharmaceutical and food production purposes. Moreover, *in vitro* studies showed that many pesticides are also potential endocrine-disruptors (Soto et al., 1994). Natural hormones and their synthetic analogues possess much higher endocrine-disrupting activity than phyto or industrial EDCs, sometimes differing in some orders of magnitude (Segner et al., 2003). However, the last ones are found in higher concentrations in the environment.

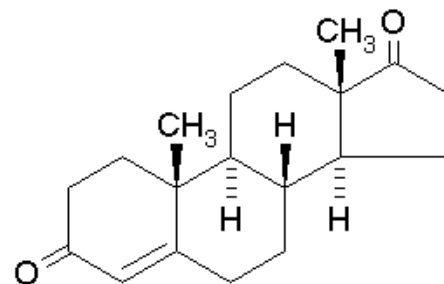
Effluent waters of sewage treatment plants (STPs) are the most reported source of EDCs in aquatic ecosystems. In case of STPs receiving predominantly domestic effluent, the majority of the endocrine-disrupting activity is originated from human hormones. This activity is ascribed to be of estrogenic and androgenic origin. Both androgens and estrogens are secreted as conjugates, which make them more water soluble and also inactive as hormones (Guengerich, 1990). However, those conjugates are cleaved by microorganisms present in STPs, releasing hormonally active substances (Ternes et al., 1999). Concentrations of EDCs found in STPs effluent waters in the United Kingdom varied from undetectable to 13 ng L⁻¹ of estradiol equivalents and undetectable to 143 ng L⁻¹ of dihydrotestosterone equivalents. Other researchers found similar values in Germany and Israel (Kirk et al., 2002). Decreases in EDCs concentrations normally occur after processes used for the clean up of the wastewater. Most of the reduction is observed during the secondary (biological) treatment. The efficiency in removing EDCs depends on the treatments used and adequate functioning of the STPs. In case of modern plants, more than 90 % of the residues are usually removed (Körner et al., 2001).

Industrial wastewaters are also considered an important source of EDCs. High estrogenic activities (from 10 to 1.200 ng estradiol equivalents kg⁻¹) were detected in sediment samples from an industrialized area in the Czech Republic (Hilscherova et al., 2002). Among the EDCs found in these samples were derivatives of alkylphenol polyethoxylates, which are non-ionic detergents of widespread application in industrial processes. In spite of the efforts to reduce the use of non-ionic surfactants, these compounds are often detected in effluent waters in concentrations around 5 µg L⁻¹ (Blackburn et al., 1999; Spengler et al., 2001). Additionally, high androgenic activities were observed in rivers receiving wastewater from paper mills. This activity probably originates from products of microbial transformation of phytosteroids released in the effluent water (Jenkins et al., 2003; Parks et al., 2001).

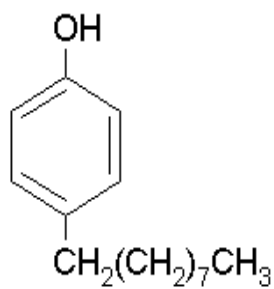
Although there are evidences of the association of livestock farming with the androgenic activity detected in raw sewage and receiving waters (Shore et al., 1993), the role of livestock as a source of EDCs has been little investigated. However, residual hormones that reach soils or waters via animal excrement may also cause problems to wild and human health. Lange et al. (2002) estimated an overall hormone secretion, by farm animals in the USA, of at least 330 tons per year. Besides, the use of anabolic agents for growth promotion has become a matter of concern. Cattle treated with diethylstilboesterol secreted about 65 % of the parent compound (Rumsey et al., 1977). Hormone residues could be detected in a soil, 8 weeks after fertilization with dung or manure of animals treated with trenbolone acetate (Schiffer et al., 2001). Most of these residues were bounded to the organic matter of the soil and only a small fraction passed through the soil column (Schiffer et al., 2004). Nevertheless, as shown by Petersen et al. (2001) EDCs from manured lands can move to surface water and ground water.

17 α -Ethinylestradiol β -Estradiol

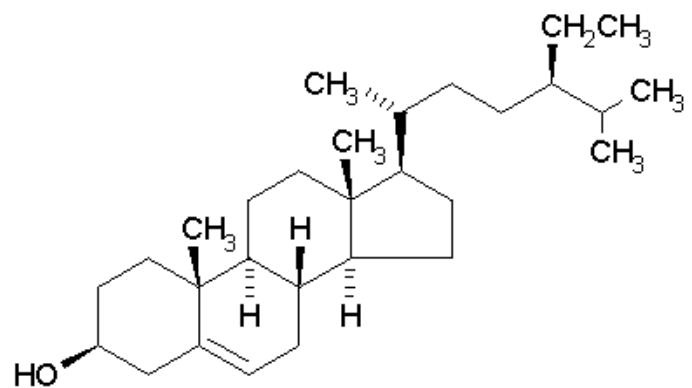
Testosterone



Androstenedione



4-nonylphenol

 β -Sitosterol**Figure 1:** Structural formula of endocrine-disrupting chemicals commonly detected in aquatic ecosystems.

1.1.2. Effects of EDCs on aquatic organisms

Many reports have been published showing effects of EDCs on vertebrates in different aquatic systems. Most of these studies describe the feminilization of male fish in rivers or lakes receiving effluent water from STPs. These males had a pronounced increase of the plasma concentration of the yolk precursor protein vitellogenin, normally only associated with sexually mature females (Purdom et al., 1994), indicating an estrogen-dependent stimulation of hepatic biosynthesis. Experiments with zebrafishes showed that the induction of vitellogenin occurred by concentrations higher than 1.67 ng L^{-1} ethinylestradiol or $375 \text{ } \mu\text{g L}^{-1}$ bisphenol A (Segner et al., 2003). Länge et al. (2001) observed that at a dosage of 4 ng L^{-1} ethinylestradiol male fathead minnows fail to develop normal secondary sexual characteristics. Moreover, androgen-induced changes of female fishes were documented for North America and Europe. In the River Fenholloway, 80 % of the mosquitofish found downstream a paper mill were completely masculinized. They displayed an anal fin which had a greater number of segments and was elongated, resembling a male-like gonopodium (Parks et al., 2001).

Less information is available about the effects of EDCs on invertebrates. Limited knowledge regarding the endocrine system of invertebrates has hampered the assessment of endocrine-disrupting effects on these animals. Segner et al. (2003) observed effects of ethinylestradiol, bisphenol A and octylphenol on the development and reproduction of coelenterates, crustaceans, insects and molluscs. Many developmental and reproductive parameters were affected by the tested estrogens. For example, 100 days exposure to a dosage of $1 \text{ } \mu\text{g L}^{-1}$ 17β ethinylestradiol caused an increase of population size of the crustacean *Gammarus pulex*. Furthermore, the sex ratio was biased in more than 2:1 in favor of females.

Effects of EDCs in other non-target organisms, especially microorganisms, have been little investigated. EDCs may affect microorganisms in two divergent ways: by damaging microbial cell physiology or by supporting microbial growth as a carbon source. Okai et al. (2004; 2000) observed an inhibition of growth of *S. cerevisiae* and *E. coli* and showed that it was associated to nonylphenol-induced formation of radical oxygen species. Although free radicals can damage nucleic acids, proteins and lipids in the cell, nonylphenol-induced radicals preferentially attacked lipids in the membrane. Moreover, hydrophobic substances can accumulate in the membrane, causing changes on the lipid composition (Donato et al.,

2000) and disturbing respiration related enzyme activities (Donato et al., 1997). On the other hand, EDCs can be used as carbon sources by microorganisms. Fujji et al. (2002) isolated a Gram-negative bacterium from activated sludge able to transform estradiol in very low molecular mass compounds (e.g. CO₂) or organic acids. Also fungi are shown to degrade EDCs. *Fusarium proliferatum* metabolized 97 % of the ethinylestradiol added to the medium, leading to the formation of more polar substances. The toxicity and disrupting activity of these degradation products still need to be investigated (Shi et al., 2002).

1.2. Trenbolone: a model of EDC with androgenic activity

Trenbolone (17 β -hydroxy-4,9,11-androstatrien-3-one or TBOH) was chosen as a model of EDC with androgenic activity for the present study. This synthetic steroid hormone is the effective ingredient of many implant preparations used for growth promotion in cattle production. TBOH is licensed in many meat-exporting countries outside the European Union, such as USA.

Interactions of TBOH with androgen, progestin and glucocorticoid receptors (Meyer and Rapp, 1985) produce an anabolic effect 8 to 10 times stronger than its natural analogue testosterone (Neumann, 1976). It is administered subcutaneously as trenbolone acetate, which is readily deacetylated in the blood circulation to β -TBOH, the most hormonally active form. In the liver β -TBOH is further metabolized to trenbolone and α -TBOH, which strongly decrease the compound's biological activity. A portion of the active androgen, β -TBOH, is excreted by the cattle along with its metabolites, primarily, 17 α -trenbolone and trenbolone (Pottier et al., 1981).

Mutagenicity tests using *Salmonella thyphimurium* detected 0.2 revertants per nmol of TBOH. Apparently TBOH can penetrate the bacterial membrane and react with DNA, resulting in pro-mutagenic lesions. The testable dosage range was limited due to a severe bactericidal activity at concentrations higher than 60 μg per plate ($\pm 3 \mu\text{g mL}^{-1}$), which difficult the interpretation of the test (Lutz et al., 1988). In addition it was found that TBOH forms covalent bounds with cell proteins (Hoffmann et al., 1984). According to Lutz et al. (1988) TBOH residues should represent a low genotoxic risk for meat consumers. Although many studies have been performed on the safety of TBOH for animals and consumers, its effects on soil and aquatic organisms is still unknown.

1.3. Possible assays in ecotoxicological approaches for the investigation of microbial communities

1.3.1. Role of benthic microorganisms in aquatic ecosystems

Sediment microorganisms are crucial for the degradation of organic matter and cycling of nutrient in aquatic ecosystems (Rheinheimer, 1992). Therefore, changes of microbial community structure or functions in sediments caused by pollutants may have consequences on higher trophic levels and on the overlying water column. Patton et al. (2004) detected high concentrations of Pb in protozoa feeding on *Pseudomonas putida*, which previously accumulated this metal. An enrichment of resistant bacteria populations, able to accumulate high concentration of a certain pollutant, may lead to the intoxication of predators. Moreover, as some animals graze on very specific microbial groups, their replacement by resistant microorganisms can cause starvation of predators (van Beelen and Doelman, 1997). Similarly, the replacement of bacterial groups, which carry out important biogeochemical processes, may have serious consequences, as resistant-microorganisms often fail to perform specific ecological functions (van Beelen and Doelman, 1997). In fact, contamination of sediments with heavy metals or organic pollutants was shown to impair microbial processes, such as methanogenesis (Capone et al., 1983; Pedersen and Saylor, 1981).

1.3.2. Ecotoxicological approaches for investigation of effects of pollutants on microorganisms

Effects of pollutants on microorganism can be assessed by single-species or multiple-species tests. The choice of the test to be employed depends on the questions addressed. Although single-species tests have the advantage of giving results on the short-term effects of pollutants under standardized conditions, the used species are not always representative for the investigated aquatic system (Koivisto, 1995). Processes which affect the availability of the chemical in a given environment are minimized in single-species tests. Furthermore, these tests cannot accurately predict effects on species competition or prey-predator interactions (Wong and Dixon, 1995). On the other hand, holistic approaches are not suitable to describe isolated components of the system. Nevertheless, multiple-species tests are closer to natural conditions than single-species assays. An adequate assessment of the potential risk of

pollutants should include different trophic levels present in an ecosystem (Severin-Edmaier, 2000).

1.3.3. Microbial cell number and biomass as an ecotoxicity indicator

The determination of cell numbers is one of the most used approaches to study changes on microbial communities caused by pollutants. However, before counting, the cells must be extracted from sediments. The efficiency of many extraction methods have been discussed (dos Santos and Casper, 2000), but probably none of them is able to recover all cells attached to sediments particles and/or organic matter.

Although plate counts give relative values in the presence of a toxicant compared to a control, similar cell numbers do not necessarily indicate lack of effects, but may reflect a selection of resistant organisms. Müller and collaborators (2001b) showed that cultivable bacterial populations from soils contaminated with mercury were structurally less diverse and contained a higher proportion of resistant fast growing forms than observed in control. The use of cultivation dependent techniques has been criticized as only a small fraction of bacteria living in natural environments is able to grown in culture medium (Amann et al., 1995). However, some authors claimed those organisms as representatives of the metabolic active fraction of the community (Ellis et al., 2003) and, therefore, assumed that plate counts are more sensitive than total cell counts for the detection of effects of pollutants. More investigation should be undertaken in order to confirm this hypothesis.

Direct counts do not display selection problems observed for the culture methods. Special staining techniques, such as acridine orange, may allow estimations of the overall bacterial activity by discriminating between metabolic active and non-active cells. Fabiano et al. (1994) suggested that low percentage of metabolic active cells may indicate toxic effects, such as observed for heavy metals contamination. However, since many staining procedures were not satisfactory or only slightly satisfactory in distinguishing between viable and dead cells (Maukonen et al., 2000), the usefulness of these techniques has earned criticism.

The total microbial biomass is also an important parameter for the detection of effects of pollutants on microorganisms. As this approach does not distinguish among microbial groups, it reflects the sum of the responses of different microorganisms present a sediment sample.

More sensitive species may lyse. Resistant or tolerant organisms can grow more intensively due to the reduction in competition for available nutrients and organic matter released from decaying microorganisms (van Beelen and Doelman, 1997). Therefore, some authors have considered that absolute biomass concentrations alone are not very useful for the estimation of sediment toxicity (Eismann and Montuelle, 1999). They assume that the relationship between overall microbial activities and biomass better represents effects of pollutants on microbial communities.

1.3.4. Structural diversity of microbial communities: role and determination

In theory, species diversity should be a more sensitive indicator of pollutant toxicity than sumparameters like respiration, as changes in species composition do not necessarily affect process rates. However, side effects of chemicals, such as pesticides, on microorganisms are commonly assessed by functional parameters, neglecting the importance of microbial structural diversity; even though the reduction of microbial diversity may affect the ability of the system to respond to perturbations (Johnsen et al., 2001). Practically, methodological limitations hinder detailed descriptions of microbial diversity, which may difficult the detection of effects of pollutants.

Molecular techniques may provide a fundamental base to overcome inherent limitations of cultivation-dependent methods. They are based on analyses of biomarkers, e.g. DNA, lipids and proteins. Morgan and Winstanley (1997) defined biomarkers as any biological component used to indicate a useful feature of a certain organism. Among the many biomarkers proposed for investigation of microbial communities, phospholipids fatty acids (PLFA), ribosomal RNA (rRNA) and rRNA genes have been most often used for assessment of diversity in sediments.

PLFA, the main constituents of cellular membranes, are readily degraded after cell death. Therefore, PLFA are used for determination of viable biomass in sediment (White et al., 1979). Moreover, analyses of microbial fatty acids by gas-liquid chromatography coupled to mass spectrometry provide qualitative and quantitative data about different microbial groups within the community (Gattinger, 2000). Modifications of environmental conditions, e.g. temperature, may lead to changes in the cellular lipid profile. This must be taken into account when fatty acids are used for identification purposes. However, these physiological changes

can be exploited to determinate effects of environmental pollutants on microbial communities (Morgan and Winstanley, 1997).

Genes coding for rRNA are assumed to be ideal biomarkers for studies of microbial diversity due to the following reasons: 1) they are found in all living cells due to the essential function of their products in protein synthesis, 2) they can be used for determination of evolutionary relationships (Woese, 1987), 3) the presence of different levels of conservation within their sequence allows the design of primers or probes for different taxonomic levels (Kämpfer et al., 1996), 4) a large number of sequences have been stored in public data bases. Different techniques were developed to assess microbial diversity based on rRNA genes. Sequence analyses of clone libraries of rRNA gene fragments, obtained by PCR, provide qualitative information about microbial community composition. Although this may be the best approach available for the description of microbial communities in environmental samples, its laboriousness impairs the analyses of multiple samples. Fingerprint methods, such as denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP), provide a pattern or profile of the genetic diversity in a microbial community. In the case of DGGE, PCR products of the same length, but different sequences, can be separated in polyacrilamid gels containing a linear gradient of denaturants. The separation is based on the decrease of the electrophoretic mobility of partially denatured double strand DNA (Muyzer and Smalla, 1998). Fingerprints techniques allow investigations of a high number of samples. Moreover, statistical tools, such as cluster analysis or principal component analysis, can be used to determine similarities between microbial communities based on their band profiles. On the other hand, the use of rRNA-targeted oligonucleotide probes labeled with fluorescent dyes allows the in situ detection of single cells in complex environmental samples. This technique provides quantitative data about microbial groups present in natural samples (Amann et al., 1995).

1.3.5. Microbial community functions and their investigation

1.3.5.1. Overall microbial activity

Respiration is one of the most used methods to estimate overall microbial activity. Effects of pollutants on the CO₂ production can be followed in sediment samples with or without substrate amendment. The addition of substrates may facilitate the detection of pollutant

effects on microbial communities. Experiments using high amounts of substrates reflect growth kinetics, with higher CO₂ production than in natural conditions (van Beelen and Doelman, 1997). The use of radioactive labeled compounds permits the detection of microbial respiration at lower substrate concentrations, which are more similar to the conditions in the environment. As showed by Widenfalk et al. (2004) respiration may be often less sensitive to pollutants than other activity parameters. Incubation period and substrate concentration can influence the sensitivity of the test. For example, the herbicide dinoseb acetate treatment caused a reduction of short-term respiration (12 hours) whereas long-term respiration (14 days) did not show any effect (Malkomes and Wöhler, 1983). Österreicher-Cunha et al. (2003) observed that the amendment of nutrients to soils contaminated with hexachlorocyclohexane lead to the recovery of their respiratory activity.

Microcalorimetry has been used as an alternative approach for the determination of overall microbial activity in environmental samples (Ljungholm et al., 1979). This method, which is based on the detection of heat released during microbial metabolism, has been successfully used to measure microbial activity in complex systems, and is able to monitor aerobic as well as anaerobic metabolic processes (Barja et al., 1997). Therefore, microcalorimetry can be considered even more general than respiration for the estimation of microbial activity. The usefulness of microcalorimetry for the detection of effects of pollutants on microbial activity have been demonstrated in several studies (Crittter and Airoidi, 2001; Prado and Airoidi, 2001; Prado and Airoidi, 2003).

1.3.5.2. Investigation of specific microbial processes

Some microbial processes, such as nitrogen fixation, are difficultly direct quantified (van Beelen and Doelman, 1997). Indirect methods, e.g. quantification of the expression of functional genes or enzyme activities, can be used as an alternative for the investigation of microbial processes. Various methods have been used to quantify mRNA from environmental samples including: RNase protection assay, northern-blotting and RT-PCR (Sayler et al., 2001). Fleming et al. (1993) showed that naphthalene dioxygenase (*nahA*) transcript levels positively correlated with naphthalene mineralization rates, naphthalene concentration and *nahA* gene frequency. This shows that the expression of specific genes can act as suitable indicators of actual microbial process rates. However, the limited information about target

sequences, which is required for the design of primers and probes, still difficult the use of this approach.

Enzyme assays are often used to indicate effects of pollutants on environments, because they are simple and reflect important microbial functions. However, they provide information about potential and not on actual activity. The hydrolytic cleavage of polymers is usually the rate-limiting step for the decomposition of organic matter. As polymers, such as proteins, chitin and cellulose, cannot be transported through bacterial membranes, extracellular enzyme catalyze the transformation of these compounds to smaller units, which can be further metabolized in the cell (Eismann and Montuelle, 1999). Enzyme activity quantification requires incubation in aqueous suspensions containing specific substrates, which may cause changes in environmental conditions (van Beelen and Doelmann, 1997). The kinetic behavior of clay- or humus-sorbed enzymes often differs from the free enzymes. Therefore, release of enzymes attached to sediment particles will probably interfere with their activity (Kuhbier et al., 2002).

1.3.6. Combining microbial community structure and function

Some techniques provide information about the function of microbial groups, present in environmental samples, under conditions that approach in situ conditions. They are very useful for the investigation of interactions of pollutants with microorganisms, e.g. for the assessment of in situ degradation. The combination of FISH with microautoradiography allows the visualization of metabolic active microorganisms by the detection of cells which up take radiolabeled substrates (Lee et al., 1999). (Urbach et al., 1999) used the BrdU-immunochemical detection method for the identification of populations that grow in response to a specified or measured stimulus. 5-deoxybromouridine (BrdU), an analogue of thymidine, is shown to be taken up and incorporated into the DNA during its synthesis. Therefore, this compound can be used to label replicating microbial cells within a population or a community. Antibody against BrdU allows the detection and separation of these cells (or their DNA) from non-labeled cells. Yin et al. (2000) successfully used the BrdU-approach to study the diversity of bacterial communities growing on substrates added to the soil. BrdU immunodetection has the great advantage of not requiring the use of radiolabeled substrates. In spite of the enormous potential of the method this is the first time in which the BrdU immunodetection was used to address ecotoxicological enquiries.

1.4. Objectives

The present thesis has been a part of a joint project between the Institute of Ecological Chemistry, the Institute of Soil Ecology and the Institute of Biometry and Biomathematics of the GSF National Research Center for Environment and Health, Neuherberg. The aim of the project, started in 1998, was to evaluate potential effects (direct or indirect) of EDCs on zooplankton, phytoplankton, nematodes and benthic microbial communities of aquatic model systems. Previous experiments using the model substances, nonylphenol and ethynilestradiol, demonstrated the usefulness of controlled release approach for the detection of long-term effects of estrogens on the cited organisms. As androgens are also found in aquatic environments, a new experiment was designed in which β -TBOH was used as model substance. This allowed not only the investigation of effects of androgens on the different organisms studied, but also the comparison between their responses to estrogenic and androgenic active substances.

Based in the few data available in the literature, it is expected that endocrine-disruptors should not affect non-target organisms, such as microorganisms, at the concentrations usually found in the environment. In order to test this hypothesis, possible effects of β -TBOH on the structural and functional diversity of benthic microbial communities were investigated.

The main objectives of the thesis were:

- I) to detect changes of bacterial and archaeal community structure as well as total genetic diversity caused by β -TBOH treatment.
- II) to characterize bacterial communities from β -TBOH treated and untreated sediments.
- III) to compare the sensitivity of total and active bacterial communities for the detection of effects of β -TBOH.
- IV) to investigate effects of β -TBOH on ecologically relevant functional parameters of the studied microbial community.

2. Materials and Methods

2.1. Information about the model substance

β -TBOH was used in this study as a model of endocrine-disrupting chemical with androgenic activity. Information about the structural formula and physical-chemical characteristics of β -TBOH are given below (figure 2 and table 1).

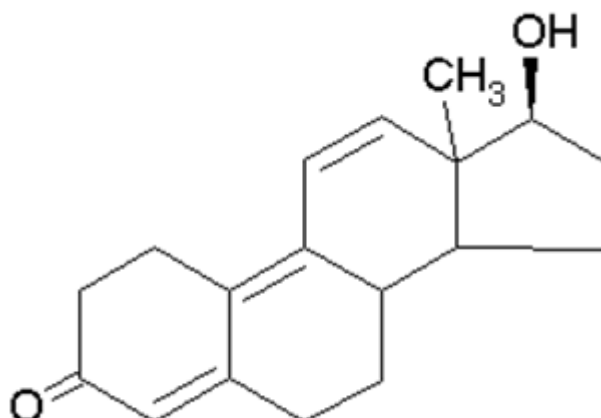


Figure 2: Structural formula of the synthetic steroid β -TBOH.

Table 1: Information about β -TBOH (FDA, 1987)

Synonyms	17 β – hydroxyestra–4, 9, 11-trien–3-one
Molecular formula	$C_{18}H_{22}O_2$
Molecular weight	270.37
Water solubility	340-380 mg L ⁻¹
Kow	1040

2.2. Experimental design

2.2.1 Outdoor microcosm experiment

An outdoor microcosm system was used to investigate changes on zooplankton, phytoplankton, nematodes and benthic microbial communities caused by the continuous

exposure to the androgen TBOH. Data related to microbial community structure are presented in this work, for other organisms see (Jaser, 2003).

2.2.1.1. Sediment and water sampling

The water and sediment used in this experiment were collected in spring 2001 from an oligo-mesotrophic littoral area of the Lake Ammersee (Melzer et al., 1988), localized in the municipality of Schondorf, Bavaria, Southern Germany. The Lake Ammersee has 47 km² surface, 1.774 km³ volume and a maximum depth of 82.5 m (World lakes database - <http://www.ilec.or.jp/database/eur/eur-23.html>). Information about the studied sediment is given in table 2.

Table 2: Physical characteristics of the Lake Ammersee sediment.

pH	7.5
Water content	50 % of the total weight
Grain of the size fraction 0.1-0.2 mm Ø	3 %
Grain of the size fraction 0.01-0.1 mm Ø	20 %
Grain of the size fraction < 0.01 mm Ø	78 %

The water was taken using an electric pump and passed through 63 µm pore size filters to remove the zooplankton and avoid unequal distribution of these organisms among the microcosms. The sediment was collected with a shovel from the surface (0 to 20 cm) in a water column depth of 60 cm. To remove particles, such as plant pieces and stones, the sediment was pressed through a metal sieve (pore size 0.5 cm). The sieved sediment was homogenized using a cement mixer.

During the experiment run, sediment samples collected from the same location in the lake were compared to the microcosm samples in relation to their abiotic parameters and bacterial community structure.

2.2.1.2. Microcosm set up and TBOH treatment

Stainless steel containers (Ø 80 cm, height 60 cm) were filled with a 10 cm layer sediment and 200 L water. Zooplankton collected using a plankton net (pore size 63 µm) was separated from non-zooplankton organisms, quantified and equally distributed among the microcosms. The containers were kept in an artificial pond, surrounded by water, in order to avoid extreme temperature variations. Moreover, in case of heavy rainfall the roof was closed to prevent overflow. 6 replicates of control and 6 differently TBOH treated microcosms were randomly alternated in rows and named K1 to K6 and A to F, respectively (figure 3).

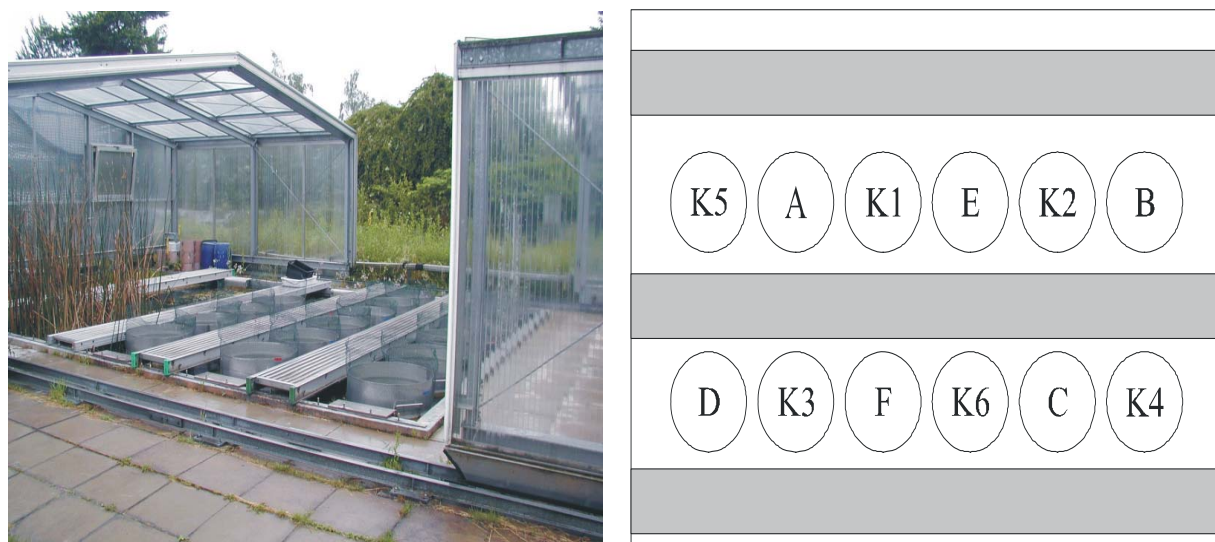


Figure 3: Photo of the microcosm facility and scheme of the microcosm arrangement (Institute of Soil Ecology, GSF National Research Center for Environment and Health, Neuherberg). K1 to K6 = replicates of control microcosms, A to F = TBOH treated microcosms.

The experiment started in May 2001 with the pre-treatment phase, which ran for 5 weeks. The treatment phase began in July and lasted for 9 weeks, followed by a period of 6 weeks without any TBOH treatment, called pos-treatment phase. 5.7 % technical TBOH (Sigma-Aldrich, Steinheim, Germany) diluted in triolein was applied to the microcosms using semi-permeable low-density polyethylene (LDPE) membranes (table 3). Perforated stainless steel boxes were used to protect the membranes against mechanical damage. The release of the chemical by this method is determined by the diffusion rate through the polymer membrane and the concentration of the substance in the reservoir, assuming that the temperature is constant and

the water phase is not close to the saturation limit (Pfister et al., 2003). Solubilizers do not affect the system as they do not pass through the membrane.

Table 3: Filling of the LDPE membranes for the controlled release of TBOH in the outdoor microcosms.

Microcosm	Exposition (in days)	Nominal length of the membranes (cm)	Area (cm)	Filling volume (mL)
A	47 ^{**}	1	5.4	0.04 [*]
	15	1	5.4	0.02 [*]
B	47 ^{**}	3	16.2	0.12 [*]
	15	3	16.2	0.06 [*]
C	47 ^{**}	11	59.4	0.44 [*]
	15	11	59.4	0.22 [*]
D	47 ^{**}	36	194.4	1.44 [*]
	15	36	194.4	0.72 [*]
E	47 ^{**}	120 (2 X 60)	648	4.8 [*]
	15	120 (2 X 60)	648	2.4 [*]
F	47 ^{**}	240 (4 X 60)	1296	9.6 [*]
	15	240 (4 X 60)	1296	4.8 [*]

*Dispersion of 5.7 % TBOH in triolein. Conclusions about the actual concentration of TBOH in water and sediment can not be determined using data in the table.

** After 47 days, the membranes were substituted.

2.2.1.3. Microcosm sampling

Three cores were weekly taken from the first 1 cm of the sediment in each microcosm using plastic cylinders (Ø 2.5 cm, height 80 cm) as illustrated below (figure 4). Samples were kept at – 20 °C until further analyses. Table 4 shows the scheme of measurements made during the experiment.

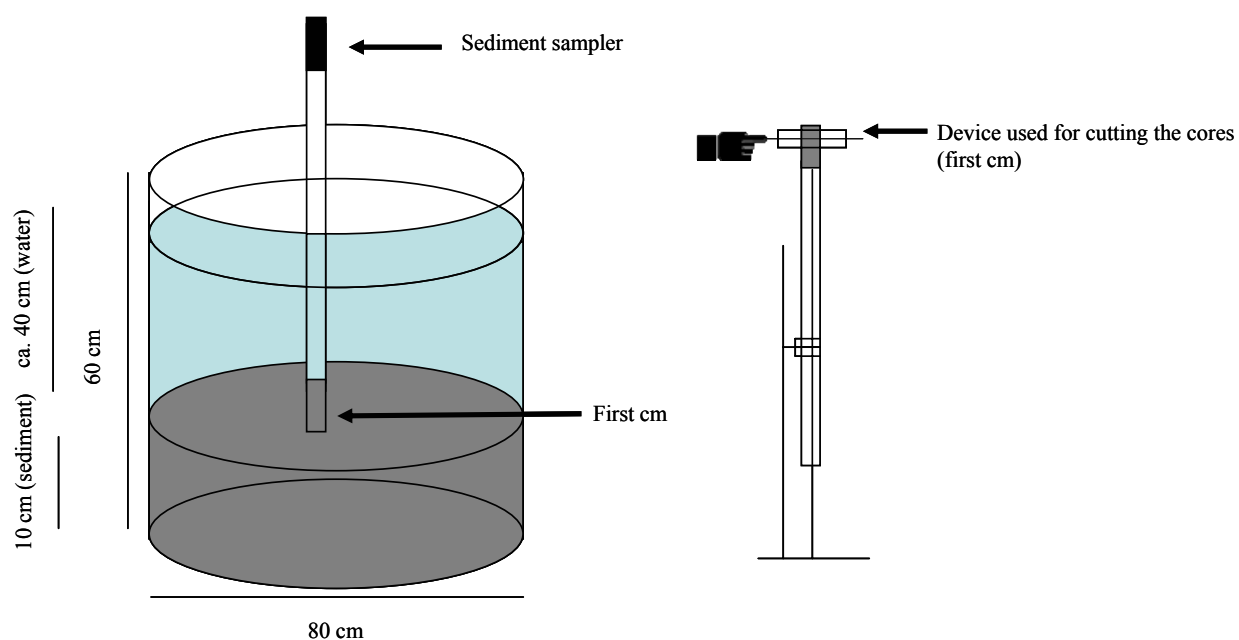


Figure 4: Diagram of the outdoor microcosm and device used for sediment sampling.

Table 4: Scheme of the measurements and analysis of sediment samples made during the outdoor experiment.

Measurement	Pre-treatment	Treatment	Post-Treatment
Redox potential	1	weekly	2
pH/temperature			
NH_4^+ , NO_3^- , PO_4^{3-}	2	weekly	3
DOC	2	2	2
16S rDNA fingerprint-bacteria	1	2	1
16S rRNA fingerprint-bacteria	1	1	1
16S rDNA fingerprint-archaea	1	1	1
16S rDNA clone library	-	1	-

2.2.2. Laboratory microcosm experiment

To investigate short-term effects of TBOH on the microbial community structure and functions a laboratory microcosm experiment was set up.

2.2.2.1. Sediment and water sampling

Sediment and water were collected from the area described above during autumn 2002. The sediment was taken with a shovel from the surface (0 to 20 cm) in a water column depth of ca. 60 cm. Stones and other particles were manually removed before mixing the sediment. Except to mixing, no further processing was done for the water.

2.2.2.2. Microcosm set up and TBOH treatment

The experiment was carried out in stainless steel containers (Ø 15 cm, height 25 cm) filled with a 10 cm sediment layer (corresponding to 3.2 kg) and 1.5 L water. In total, eight microcosms were set up, comprising four replicates of each, control and TBOH treatment. The microcosms were incubated at day light and room temperature of around 20 °C. 15 days after the experiment was set up, 47 µL of 1.5 mg mL⁻¹ technical TBOH (Sigma-Aldrich, Steinheim, Germany) in ethanol was added to 50 mL lake water and directly applied to the water layer of the microcosms. The same volume of ethanol was added to each control microcosm. TBOH was added to reach a concentration of circa 15 µg kg⁻¹, which can be found in sediment receiving high inputs of EDCs. The experiment was finished 19 days after TBOH treatment.

2.2.2.3. Microcosm sampling

The size of the microcosms did not allow the removal of sediment samples without disturbing the system. Therefore, only RAPD analyses, which require 500 mg sediment, were done during the incubation period. Samples were taken using a cut 25 mL pipettes similarly as described in 2.2.1.3. 19 days after TBOH treatment, the overlying water was removed and the first 5 cm sediment was collected, mixed and stored at -20 °C for measurement of all parameters, except the substrate utilization potential, which was determined immediately after

the end of the experiment. Table 5 shows the scheme of measurements made during the experiment.

Table 5: Scheme of the analyses of sediment samples made during the laboratory experiment.

Measurement	
NH_4^+ , NO_3^- , PO_4^{3-}	19 days after TBOH treatment
DOC	19 days after TBOH treatment
16S rDNA/rRNA fingerprint-bacteria	19 days after TBOH treatment
RAPD	before TBOH treatment 10, 14 and 19 days after TBOH treatment
Cell count	19 days after TBOH treatment
Nitrite reductase and chitinase gene detection	19 days after TBOH treatment
Substrate utilization potential	19 days after TBOH treatment
Enzyme activities	19 days after TBOH treatment

2.2.3. 5-Bromodeoxyuridine (BrdU) incorporation experiment

Sediment samples were incubated with BrdU to investigate the effects of TBOH on the active fraction (growing cells) of the bacterial community. This analogue of thymidine is incorporated into the DNA during synthesis and can be used to label metabolic active cells. Antibody against BrdU, present in single-strand DNA, allow the detection and separation of labeled cells or their DNA.

2.2.3.1. Sediment sampling and experiment set up

Sediment samples taken from the lake as described in 2.2.2.1. and were pre-incubated in humid chambers at 23 °C for 3 to 5 days. Petri dishes containing 3 g sediment were amended with 4 mg g⁻¹ of yeast extract and with or without 0.5 mg g⁻¹ TBOH and incubated at 23 °C overnight. Samples were then supplemented with 500 µL of 1.4 mM BrdU, incubated for 2 h

and immediately frozen. Labeled DNA was separated from unlabeled DNA by immunocapture and the bacterial community was analyzed by 16S rDNA-DGGE (figure 5).

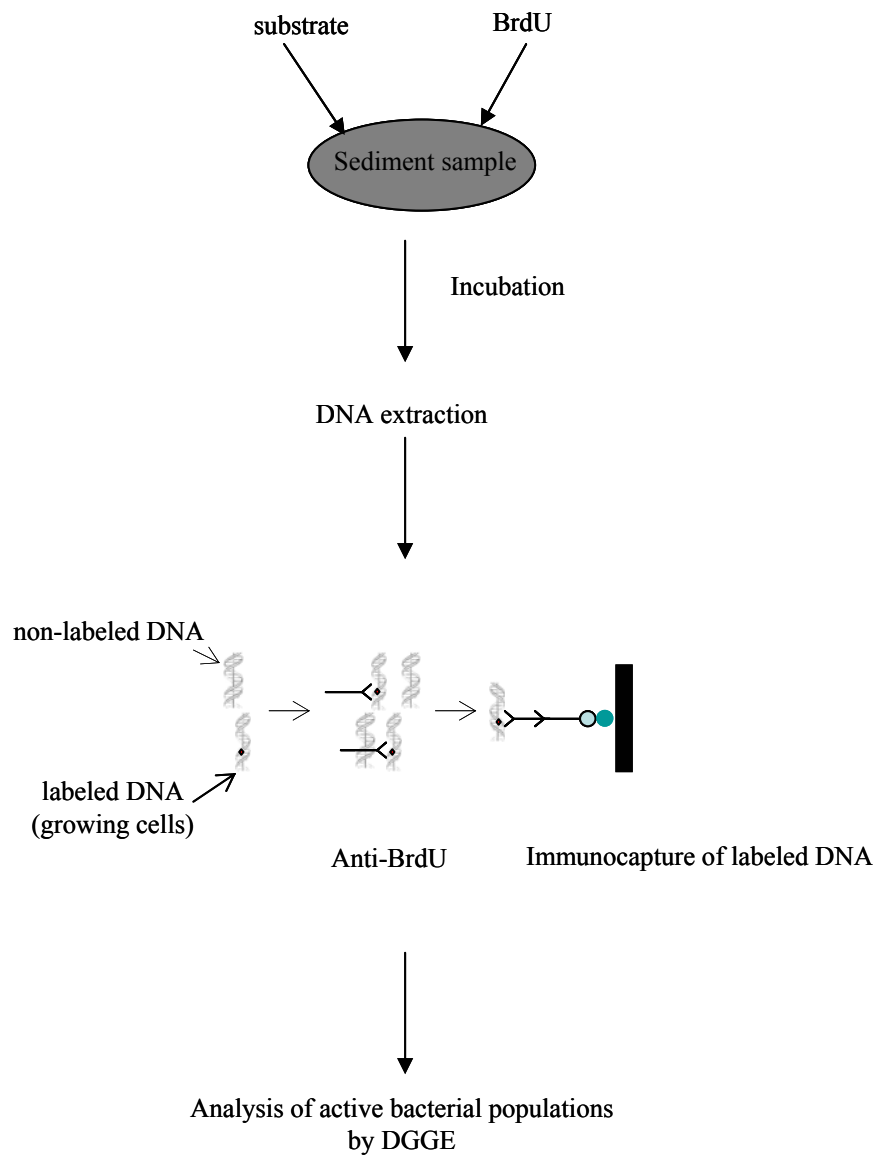


Figure 5: Diagram of the analysis of the active fraction of bacterial community by BrdU labeling and immunocapture.

2.3. Measurement of the abiotic parameters

2.3.1 Measurement of NH_4^+ , NO_3^- , PO_4^{3-}

Two grams of sediment were overhead shaken for 2 h with 18 mL of 0.01 M CaCl_2 containing 2 mg mL^{-1} sodium azide and 300 $\mu\text{g mL}^{-1}$ chloramphenicol. The extract was filtered (pore size 0.45 μm) and PO_4^{3-} , NH_4^+ and NO_3^- measured using the commercial kit NANOCOLOR (Marcherey-Nagel, Düren, Germany), as described by the manufacturer.

2.3.2 Measurement of DOC

For the DOC measurement, 5 g of sediment were extracted by overhead shaking for 45 min with 10 mL of 0.01 M CaCl_2 . The filtered extract (pore size 0.45 μm) was acidified to remove carbonates and quantified in a total organic carbon analyzer (TOC 5050, Shimadzu, Kyoto, Japan).

2.3.3. Measurement of pH, temperature and redox potential

The temperature, pH (TA 197 pH, WTW, Weilheim, Germany) and redox potential (SenTix[®] ORP, platin sensor, WTW) were measured in situ in the first cm of the sediment surface using the ProfiLine pH 197 (WTW).

2.4. Analyses of the microbial community

2.4.1. Buffers and media

2.4.1.1. 0.1 M Phosphate buffered saline (PBS)

Na_2HPO_4	10.9 g
NaH_2PO_4	3.2 g
NaCl	90 g

Volume was adjusted to 1 L using dH_2O

pH was adjusted to 7.2

2.4.1.2. Tris acetic acid EDTA buffer (TAE)

Tris base	242 g
EDTA	18.6 g
Acetic acid	57.1

Volume was adjusted to 1 L using dH₂O

pH was adjusted to 8.0.

2.4.1.3 BW buffer

Tris-HCl	1.576 g
EDTA	3.72
NaCl	116.8 g

Volume was adjusted to 1 L with dH₂O

pH was adjusted to 7.2

2.4.1.4. Nutrient Broth (Fluka, Seelze, Germany)

Peptone from meat	5 g
Meat extract	3 g

Volume was adjusted to 1 L with dH₂O

pH 7.0

2.4.1.5. LB medium

Tryptone	10 g
NaCl	5 g
Yeast extract	5 g

Volume was adjusted to 1 L with dH₂O

pH 7.5

2.4.2. Cell Counts

Bacterial cells were extracted from 2 g of sediment with 18 mL of 0.1 M PBS by overhead shaking for 30 min. Sediment slurries were centrifuged at 800 x g for 10 min, the supernatants

transferred to new tubes and centrifuged at 5000 x g for 10 min. Sediment pellets were re-extracted with 18 mL of 0.5 % sodium cholate. The last procedure was repeated using a solution 0.5 % sodium cholate and 0.1 % polyethylene glycol (PEG). Pellets were resuspended in 18 mL of 0.5 % sodium cholate, 0.01 % SDS and 1 % PEG and kept for 10 min in ultrasonic bath. Supernatants were pooled, centrifuged and the pellet was resuspended in 2 mL 0.1 M PBS. The cell density was estimated by direct count (Porter and Feig, 1980).

Polyethylene carbonate filter (0.2 µm pore size) washed with sterile 0.1 M PBS was used to filter 100 µL of the suspension cell of the pooled supernatant. Half of the filter was covered with 100 µL of 4'-6-diamidino-2-phenylindole (DAPI) solution (1 µg L⁻¹ in dH₂O) and incubated in the dark for 10 min. The filter was carefully washed with sterile 0.1 M PBS and dried at the dark. The cells were quantified under UV by epifluorescence microscopy (Axioplan, Zeiss, Oberkochen), with a DAPI fluorescence emission filter (filter 01, Axioplan, Zeiss, Oberkochen). The average cell count was determined from 20 separate field counts, and the cell density was then inferred from the filter and field areas. Sterile dH₂O was processed as described above and used “zero sample”.

$$\text{Number of cells} = \frac{(\text{total of cells counted in the sample}) - (\text{zero sample})}{\text{number of fields}} \times M \times V$$

M = microscope factor

V = dilution factor

2.4.3. Nucleic acids extraction

DNA and RNA were co-extracted from 500 mg sediment as described by Griffiths et al. (2000). 24 µL of each extract were treated with 3 U of RQ1 RNase-free DNase (Promega, Madison, USA) for 30 min at 37 °C. Digestion was stopped by adding 3 µL of RQ1 DNase stop solution and the product was reverse transcribed (2.4.4.). The efficiency of the DNA digestion was checked by direct amplification of RNA templates without reverse transcription. An aliquot of the extract was not digested and was used as DNA template.

The quality and quantity of the DNA was evaluated in 1 % agarose gels in 1x TAE stained in ethidium bromide (Sambrook et al., 1989). One kb or 100 bp ladders (MBI Fermentas, Vilnius, Lithuania) were used as molecular weight standards.

2.4.4. cDNA synthesis

cDNA synthesis performed using the Omniscript RT kit (Qiagen, Hilden, Germany). The reaction mixture contained: 1 x RT buffer, 500 μ M from each dNTP, 10 pmol of the reverse primer 1401 or hexamer primer, 10 U of RNase out recombinant ribonuclease inhibitor (Gibco BRL, Karlsruhe Germany), 4 U of omniscrypt reverse transcriptase, 5 μ L template and RNase free water to a final volume of 20 μ L. RT reactions were carried out for 90 min at 37 °C.

2.4.5. Incorporation of BrdU by pure bacterial cultures

Little information is available about the ability of different bacterial groups to up take and incorporate BrdU into their DNA. Therefore, the bacterial strains listed below, which are representatives of some of the major bacterial groups, were tested for their ability to incorporate BrdU.

- <i>Azospirillum brasiliense</i> Sp 7 (DSMZ 1690)	α -Proteobacteria	Gram-negative
- <i>Burkholderia cepacia</i> (DSMZ 7288)	β - Proteobacteria	Gram-negative
- <i>Pseudomonas fluorescens</i> (DSMZ 50090)	γ - Proteobacteria	Gram-negative
- <i>Arthrobacter citreus</i> BI 90 (Institute of Soil Ecology culture collection)	Actinobacteria	high GC Gram-positive
- <i>Cytophaga</i> sp (DSMZ 3661)	<i>Bacteroidetes</i>	CFB group bacteria Gram-negative
- <i>Bacillus subtilis</i> (DSMZ 10)	<i>Firmicutes</i>	Gram-positive

The bacteria were grown in NB at 30 °C and 150 rpm. Growth curves were determined by measuring the optical density at 600 nm. For the BrdU incorporation experiment each bacterial culture was inoculated in 5 mL NB medium and grown overnight. 70 μ L of this culture were inoculated in 7 mL medium, supplied with 100 μ L of 1.4 mM BrdU in the beginning of the log phase and inoculated for 2 h. Cells were harvested (5000 x g for 5 min) and the DNA extracted using the DNeasy tissue kit (Qiagen, Hilden, Germany). DNA extracts from bacterial cells incubated without BrdU were used as controls in the following immunocapture.

2.4.6. Immunocapture of BrdU labeled DNA

Streptavidin-coated paramagnetic beads were used to immunocapture BrdU-labeled DNA extracted from sediment samples or pure cultures. The protocol used was a modification of a previously described method (Kalle et al., 1993). Herring sperm DNA (5 mg mL^{-1}) was denatured for 5 min at 95°C , kept for 30 sec at -20°C followed by 5 min on ice. $52 \mu\text{L}$ M-270 Dynabeads (Dynal, Hamburg, Germany) were blocked with an identical volume of denatured herring sperm DNA (5 mg mL^{-1}) and incubated overnight at 4°C . $30 \mu\text{L}$ of DNA sample were denatured, mixed with $6 \mu\text{L}$ of 1:5 diluted anti-BrdU antibody (Sigma-Aldrich, Hilden, Germany), $6 \mu\text{L}$ 30 % BSA, $3 \mu\text{L}$ herring sperm DNA and $15 \mu\text{L}$ BW buffer. This mixture was incubated at 4°C overnight and subsequently for 3 h with a biotin-SP-conjugated anti-mouse antibody 1.1 mg mL^{-1} (Beckman Coulter, Villepinte, France) to a final dilution of 1:100. Blocked beads were washed 3 times with buffer BW, added to the antibody-mixture and incubated at room temperature for 2 h. Unbound DNA was removed using a magnetic separator (MPC-E, Dynal, Oslo, Norway). The pellet containing the bound DNA was washed 5 times with buffer BW. The bound fraction was released by heating for 5 min at 95°C and separated from the beads using the magnetic separator.

2.4.7. Analyses of random amplified polymorphic DNA (RAPD)

RAPD employs primers of arbitrary sequence which allow the random amplification of matching sequences within a DNA sample by PCR, resulting in a fingerprint of the total gene pool. A single 22 bp oligonucleotide (ERIC primer, modified from (Versalovic et al., 1991)) was used for the RAPD. This primer was designed to amplify short interspersed repetitive elements present in some enterobacteria. However at certain PCR conditions fragments of the genome of virtually all domains of life can be amplified (Gillings and Holley, 1997). Detailed information about all the primers used in this work is given in table 6. Reaction mixtures contained 1 x buffer (Gibco BRL, Germany), $20 \mu\text{M}$ of primer (Thermo Hybaid, Ulm, Germany), 2.5 mM MgCl_2 (Gibco BRL, Karlsruhe, Germany), 0.3 % BSA (Sigma-Aldrich, Steinheim, Germany), $200 \mu\text{M}$ of each dNTP (MBI Fermentas, Vilnius, Lithuania), 40 ng DNA template and 2.5 U *Taq* polymerase (Gibco BRL, Karlsruhe, Germany) in a final volume of $50 \mu\text{L}$. The PCR protocol consisted of one step at 95°C for 10 min, after which the enzyme was added, followed by 35 cycles at 94°C for 1 min, 53°C for 1 min, 65°C for 2 min

and a final extension at 65 °C for 10 min. The quality of all PCR products was checked in 2% agarose gels.

5 µL of purified PCR product (Qiagen, Hilden, Germany) were separated using Cleangel DNA analysis kit (Amersham Biosciences AB, Uppsala, Sweden) and stained with ethidium bromide.

2.4.8. PCR amplification of bacterial and archaeal 16S rDNA / rRNA for DGGE

A 470 bp fragment containing the hypervariable V6-V8 region of the 16S rDNA / rRNA was amplified using the primer set 968 GCf/1401r (Heuer et al., 1997) synthesized by Thermo Hybaid (Ulm, Germany). The reaction mixture contained 1 x buffer, 1.5 mM MgCl₂, 0.3 % BSA (Sigma-Aldrich, Germany), 100 µM of each dNTP (MBI Fermentas, Vilnius, Lithuania), 10 µM of each primer, 5% DMSO, 2.5 U *Taq* polymerase (Gibco BRL, Karlsruhe, Germany) and 40 ng of cDNA or DNA template, respectively, in a final volume of 100 µL. The PCR reaction was carried out as follows: initial denaturation at 95 °C for 10 min and 35 cycles at 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min (Jontofsohn et al., 2002a).

The 16S rRNA gene from archaea was amplified using the primer set W036f/ W039GCr (Leclerc et al., 2001) synthesized by Thermo Hybaid (Ulm, Germany). 40 ng DNA template were add to 98 µL PCR mixture containing 1 x buffer, 2.5 mM MgCl₂, 200 µM of each dNTP (MBI Fermentas, Vilnius, Lithuania), 0.3% BSA, 5 % DMSO, 10 µM of each primer and 2.5 U *Taq* polymerase (Gibco BRL, Karlsruhe, Germany) in a 100 µL total volume. PCR reactions were carried out as described for eubacteria, except to the annealing temperature which was 56 °C.

2.4.9. Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA / rDNA amplicons

The method permits the separation of PCR products of same size with different sequences composition. DGGE was performed, as described by (Muyzer et al., 1996), with a D-Code system (Bio-Rad Laboratories, Munich, Germany). 6 % (w/v) polyacrylamide gels (ratio of acrylamide and bisacrylamide 37:1) with a denaturant gradient from 48 % to 55 % were used

for analyzing the amplicons. The gels were run at 60 °C and 60 V for 16 h and stained with silver nitrate (Heukeshoven and Dernick, 1988).

2.4.9.1 Silver Staining

Steps	Solutions	Duration
1. Fixation	25 mL glacial acetic acid 225 mL Milli Q water	40 min
2. Washing	250 mL Milli Q water	3 times for 2 min
3. Silver reaction	0.37 g silver nitrate 0.25 mL 37 % formaldehyde 250 mL Milli Q water	25 min
4. Washing	Milli Q water	2 times for 2 min
5. Developing	6.25 g sodium carbonate anhydrous 0.25 mL 37 % formaldehyde 0.25 mL sodium thiosulfate 10 % 250 mL Milli Q water	Approximately 5 min
6. Stopping	3.65 g EDTA Na ₂ xH ₂ O 250 mL Milli Q water	10 min
7. Preserving	30 mL glycerol 75 mL ethanol	At least 30 min

2.4.10. Construction of bacterial 16S rDNA clone libraries

2.4.10.1. Amplification of the 16S rRNA gene

16S rRNA genes were amplified using the universal eubacterial primer set 27f/1401r (Heuer et al., 1997; Orphan et al., 2001) with an initial denaturation step at 95 °C for 10 min, followed by 30 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1.5 min and a final extension at 72 °C for 30 min. The reaction mixture contained 1 x buffer, 0.5 M betaine (Sigma-Aldrich, Steinheim, Germany), 200 µM of each dNTP (MBI Fermentas, Vilnius, Lithuania), 10 µM of each primer, 2.5 U *Pfu* DNA polymerase (Stratagene, Amsterdam, Netherlands) and 20 ng of DNA template in a final volume of 50 µL. Amplicons were purified using the Qiaquick PCR purification kit (Qiagen, Germany).

2.4.10.2. Production of the clones

Blunt end PCR products, generated by amplification with a proofreading polymerase, were cloned with Zero blunt[®] TOPO[®] PCR cloning kit (Invitrogen, Karlsruhe, Germany) as described by the manufacturer. The cloning reaction containing 90 ng PCR product, 1 µL salt solution, 3 µL water and 1 µL of the vector (pCR[®]II-blunt-TOPO) was incubated for 30 min at room temperature. The vector was transformed into chemically competent *E.coli* cells and incubated on ice for 5 min. The ligation of the PCR fragment disrupts the expression of the lethal *E.coli ccdB* gene permitting only the growth of positive recombinants upon transformation. All colonies were inoculated in LB medium containing 50 µL mL⁻¹ kanamycin, grown overnight and used for plasmid isolation (Qiagen Plasmid mini kit, Qiagen, Hilden, Germany). Plasmids containing inserts of correct size were selected after digestion with *EcoRI* (MBI Fermentas, Vilnius, Lithuania). Reaction mixtures containing 5 µL plasmid, 1 U *EcoRI* and 2 µL buffer O⁺ in a 20 µL total volume were incubated for 1 h at 37°C, followed by 20 min at 65°C for inactivation of the enzyme.

2.4.10.3. Screening and sequencing of the clone libraries

Clone libraries were screened by restriction fragment length polymorphism (RFLP) using the enzymes *Hin6I* and *MspI* (MBI Fermentas, Vilnius, Lithuania). Reaction mixtures containing 1.5 µL sample, 0.5 U enzyme and 1.5 µL buffer Y⁺/Tango in a 15 µL total volume were

incubated for 10 h at 37°C, followed by 20 min at 65°C. 5 to 10 µL digestion products were loaded onto a 4 % high resolution agarose gel (Qbiogene, Grünberg, Germany). Resulting RFLP patterns were clustered using the software Gelcompar II (Applied Maths, Belgium). In case of 100 % similarity, a representative clone of each RFLP group was sequenced. Sequences obtained after single run sequencing (Sequiserve, Vaterstetten, Germany) were compared with the NCBI database (<http://www.ncbi.nih.gov/BLAST/>) and aligned using the Clustal W (EMBL-EBI, <http://www.ebi.ac.uk/clustalw/>). Phylogenetic trees were viewed using the Treeview software.

2.4.10.4. Rarefaction analysis

Rarefaction analyses are useful to estimate the completeness of sampling of a community. Rarefaction curves were generated using the software Analytic Rarefaction (<http://www.uga.edu/~strata/software/Software.html>).

2.4.10.5. Nucleotide sequence accession numbers.

The 16S rDNA sequences from the clone library were submitted to GenBank and can be found as accession numbers AY793647.1 to AY793686.1.

2.4.11. Analyses of functional genes

2.4.11.1. Amplification of chitinase genes

A nested PCR approach was used to amplify the family 18 group A chitinase genes as described by (Williamson et al., 2000). 20 ng DNA template was added to 49 µL of the first-round PCR mixture containing 1 x buffer, 1.5 mM MgCl₂, 0.3 % BSA, 100 µM of each dNTP (MBI Fermentas, Vilnius, Lithuania), 10 µM of each primer (GA1f / GA1r) and 2.5 U *Taq* DNA polymerase (Gibco BRL, Karlsruhe, Germany) in a 50 µL total volume. Reactions were carried out at 95 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 60 °C for 10 sec, 72 °C for 1 min and a final extension at 72 °C for 5 min. This PCR product was diluted 1:5 and amplified as described above, using the primer set GASQf / GASQr.

In order to confirm the specificity of the primers used, a PCR product amplified from DNA extracted from sediment was cloned using the pSTBlue AccepTor Vector kit (Novagen, Madison, USA). Ligation reaction containing 1 μL AccepTor vector, 4 μL purified PCR product and 5 μL clonables 2 x ligation, premix provided by the kit, was carried out at 16 °C overnight. The transformation of the competent cells and screening of the colonies were performed according to manufacturer's instructions. Colonies containing the recombinant vector were grown overnight in LB medium containing 30 $\mu\text{L mL}^{-1}$ kanamycin and used for plasmid isolation. A plasmid containing the correct insert size, checked by *EcoRI* digestion (2.4.10.2), was sent for sequencing (Sequiseive, Vaterstetten, Germany). The correctness of the sequence was checked using the BLAST program (<http://www.ncbi.nih.gov/BLAST/>).

2.4.11.2. Amplification of nitrite reductase genes (*nirS/nirK*)

The nitrite reductase genes were amplified using primers developed by Bracker et al. (1998). PCR mixtures contained 1 x buffer, 2.5 mM MgCl_2 , 200 μM of each dNTP, 0.12 % BSA, 2 % DMSO and 10 pmol of each primer in a final volume of 50 μL . PCR cycle conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles at 94 °C for 30 sec, 56 °C or 66 °C for 40 sec (for *nirK* or *nirS*, respectively) 72 °C for 40 sec and a final extension at 72 °C for 5 min. The specificity of these primers was confirmed as described above (2.4.11.1).

Table 6: Information about the primers used in this work.

Primer set	Expected fragment size (bp)	Reference	Sequence (5'-3')
968fGC*/1401r	470	Heuer et al., (1997)	f - AAC GCGAAGAACCTTAC r - CGG TGT GTA CAA GAC CC
27f/1401r	1390	Orphan et al., (2001)	f - AGA GTT TGA TCC TGG CTC AG
W036f/W039rGC	618	Leclerc et al., (2001)	f - CTC CCC CGC AA TTC CT r - TCC AGG CCC TAC GGG G
GA1f/GA1r	ND	Williamson et al., (2000)	f - CGT CGA CAT CGA CTG GGA RTD BCC r - ACG CCG GTC CAG CCN CKN CCR TA
GASQfGC/GASQr	ND	Williamson et al., (2000)	f - CGT CGA CAT CGA CTG GGA r - ACG CCG GTC CAG GG
nirS1f/nirS6r	890	Braker et al., (1998)	f - CCT AYT GGC CGC CRC ART r - CGT TGA ACT TRC CGG T
nirK1f/nirK5r	514	Braker et al., (1998)	f - GGM ATG GTK CCS TGG CA r - GCC TCG ATC AGR TTR TGG
ERIC		Modified from Versalovic et al., (1991)	ATG TAA GCT CCT GGT GAT TCA C
Hexamer			NNN NNN

f = forward primer, r = reverse primer.

*GC clamp attached to the 5' end of the primer = CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G (Muyzer et al. 1996).

ND, not defined in the original paper.

Degenerate alphabet, M = C or A, R = A or G, Y = C or T, S = C or G, K = G or T, D = A or G or T, B = C or G or T, N = A or T or G or C

2.5. Microbial activity measurements

2.5.1. Enzymatic activities

The method is based on hydrolyses of methylumbelliferyl-substrates leading to the release of a fluorescence compound (methylumbelliferone), which can be quantified using a fluorimeter. Filtrated suspensions (pore size 80 μm), obtained after 15 min overhead shaking of 4 g of sediment in 16 mL sterile Milli-Q water, were used in the assays. The chosen substrates 4-methylumbelliferyl- β -D-glucoside, 4-methylumbelliferyl- β -D-cellobioside, 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide, 4-methylumbelliferyl-phosphate and L-leucine-7-amido-4-methylcoumarin (Sigma-Aldrich, Steinheim, Germany) target a wide range of enzymes involved in the hydrolysis of C, N and P compounds (Marx et al., 2001). Optimum conditions (substrate concentration, sediment suspension dilution and incubation time) were established for each substrate and maintained during the experiment. The reaction mixtures contained 200 μM substrate and 50 μL sediment suspension in a final volume of 150 μL , adjusted with sterile Milli-Q water. For all enzyme assays a 1:10 dilution of sediment suspension was used, except for phosphomonoesterase and N-acetyl-glucosaminidase, where 1:100 and 1:5, respectively, were optimal. Except for phosphomonoesterase, which was incubated for 30 min, all reactions were incubated for 3 h at room temperature. Reactions were stopped with 75 μL 100 % ethanol and alkalinized with 25 μL of 2.5 M Tris pH 11. Fluorescence intensity was measured at an excitation of 390 nm and an emission wave length of 460 nm in a microplate fluorimeter (FluostarII, BMG Labtech, Offenburg, Germany). Two samples were extracted from each microcosm and measured in 5 replicates. The results were calculated in nmol of released substrate per gram of dry sediment, per hour using calibration curves derived from methylumbelliferone (MUF) or aminomethyl-coumarine (AMC).

2.5.2. Substrate utilization potential

BIOLOG[®] GN2 plates (BIOLOG[®], California, USA), each containing 95 individual carbon substrates and one negative control well, were used to assess metabolic versatility and possible adaptations of the microbial communities to TBOH. A 125 μL volume of each bacterial suspension (see 2.4.2.) was added per well on the micro plates. 6 plates were used for each microcosm, 3 containing 100 ng mL^{-1} TBOH and 3 controls. The optical density ($\lambda = 590 \text{ nm}$) was measured 16, 40, 88 and 184 h after incubation at 25 $^{\circ}\text{C}$, using a microplate

reader (Spectra max 340, MWG-Biotech, Ebersberg, Germany). A well was considered positive when 2 out of 3 replicates had positive values. Plates were expressed as an index of color development in each of the wells as reported elsewhere (Ibekwe et al., 2001). Results from the 4 replicates of control and treated microcosms were pooled and presented as median.

2.5.3. Microcalorimetry

Calorimetry measurements were carried out in the microcalorimeter 2277 Bio activity monitor (LKB Bromma, Stockholm, Sweden). Sediment samples were pre-incubated in a humid chamber at 23 °C for 3 to 5 days. Sediment amended with 4 mg g⁻¹ yeast extract and without or with 15 µg kg⁻¹ TBOH were introduced in a glass ampoule, hermetically sealed and the measurement started immediately. Vials containing sediment amended with yeast extract were used as control. The microcalorimetric measurements were performed at constant temperature of 23 °C for at least 17 h and expressed as µW h⁻¹. Biomass C was determined using the lowest rate of heat production within the first 4 h after yeast extract amendment and using the relationship of Sparling (1983):

$$1 \text{ g biomass C} = 180.05 \text{ mW}$$

2.6. Statistical analysis

All data were analyzed with SPSS 11.5 (SPSS Inc., USA). The distribution of the data was examined using the Kolmogorov-Smirnov test and by visual analysis of histogram graphs. Significant differences ($p < 0.05$) were determined using the Mann-Whitney-U-test.

Gel images were analyzed with the program Gelcompar II (Applied Maths, Belgian), using the Dice coefficient and the unweighted pair group clustering method with arithmetic averages (UPGMA).

3. Results

3.1. Effects of TBOH: outdoor microcosm experiment

3.1.1 Abiotic parameters

Redox potential, temperature and pH were measured in situ in the first cm (0 to 1) of the surface of the sediment layer to follow the development of the microcosms along the incubation period.

As expected, redox potential numbers, determined at the same day in the different microcosms, showed some variation (figure 6). Values between -138 and 5 mV were detected along the experiment. Redox potential of TBOH treated microcosms were in the same range of the control microcosms. The median calculated from all microcosms varied from -30 to -62 mV.

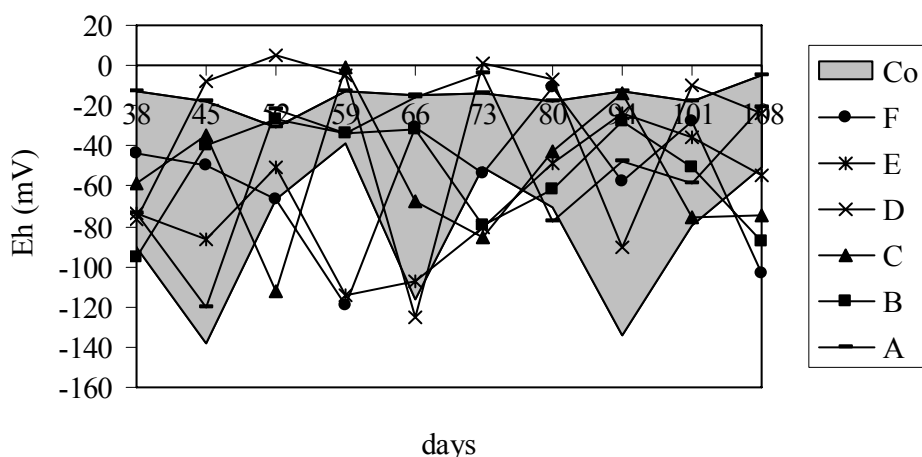


Figure 6: Redox potential data measured in the course of incubation in the first cm (0 to 1) of the surface of the sediment layer from the outdoor microcosms. Co = minimum and maximum values from control microcosms, A to F = TBOH treated microcosms.

Sediment temperature values were very similar in all microcosms, with a maximum variation detected at sampling dates 38 and 52 (figure 7). These values changed according to season, with maximum and minimum temperatures of 21 and 12 °C observed in summer and autumn, respectively. Extreme temperatures were not observed.

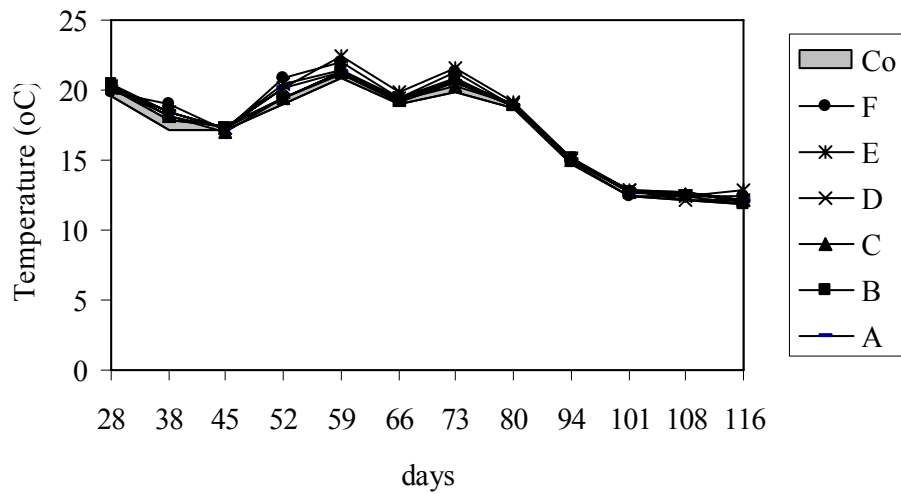


Figure 7: Temperature data measured in the course of incubation in the first cm (0 to 1) of the surface of the sediment layer from the outdoor microcosms. Co = minimum and maximum values from control microcosms, A to F = TBOH treated microcosms.

The pH of the sediment was alkaline throughout the experiment, with values ranging from 7.0 to 9.5. The values of treated microcosms did not differ from the control microcosms (figure 8)

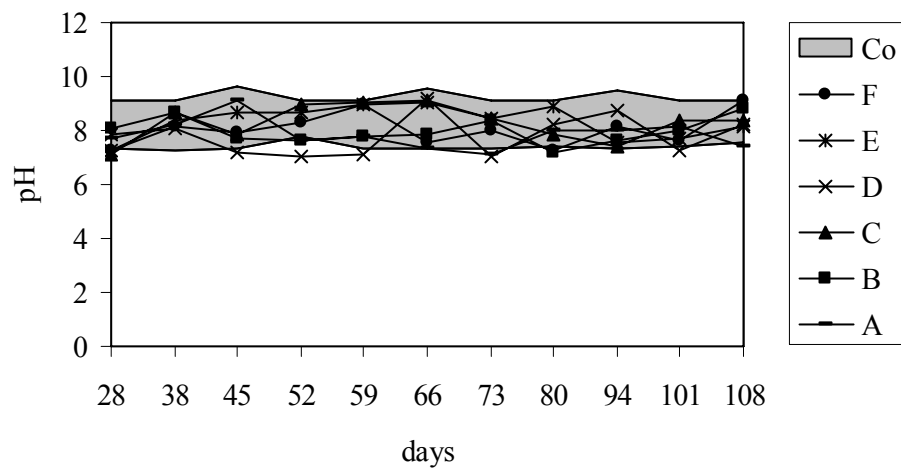


Figure 8: pH data measured in the course of incubation in the first cm (0 to 1) of the surface of the sediment layer from the outdoor microcosms. Co = minimum and maximum values from control microcosms, A to F = TBOH treated microcosms

For in situ conditions, redox potential, temperature and pH from the Lake Ammersee were measured in the first cm of the sediment surface, once during the microcosm experiment. The redox potential and temperature values measured in the lake at 25.07.01 were slightly higher than the mean values obtained at the same period in the microcosms (day 52 or 26.07.01). The pH of the sediment in the lake was 7.5, similar to the mean value of 8.0 detected in the microcosms (table 7).

Table 7: Comparison between redox potential (at measured pH), pH and temperature values of the sediment surface of the Lake Ammersee and outdoor microcosms. Numbers below represent mean values of 3 measurements made on the lake at 25.07.01 and mean of values obtained for all microcosms during sampling day 52 (26.07.01). \pm = standard deviation.

	Redox potential (mV)	Temperature ($^{\circ}$ C)	pH
Lake Ammersee	-28 ± 4.3	21.9 ± 0.09	7.5 ± 0.07
Microcosms	-49 ± 31	19.6 ± 0.6	8.0 ± 0.5

NH_4^+ , NO_3^- , PO_4^{3-} and DOC were quantified from samples collected from the first cm (0 to 1 cm) of the surface of the sediment layer. Measurements of all parameters were done once a week, except to the DOC, which was measured twice in each phase of the experiment.

The amount of NH_4^+ quantified in the TBOH treated microcosms was in the same range of the controls. In the first 45 days the values varied between 3.5 and 8.5 mg per kg of sediment. At the day 52, NH_4^+ concentrations reached a maximum of 19.6 mg kg^{-1} . After 66 days the values tended to decrease (figure 9).

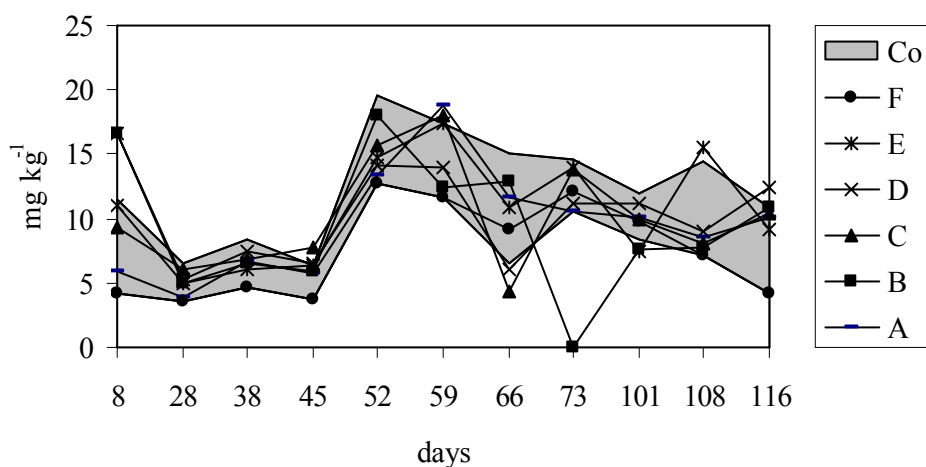


Figure 9: Course incubation measurements of NH_4^+ . Cores sampled from the first cm (0 to 1) of the surface of the sediment layer of the outdoor microcosms. Co = minimum and maximum values of control microcosms, A to F = TBOH treated microcosms.

NO_3^- concentrations detected in TBOH treated microcosms did not differ from the controls. The highest NO_3^- values were quantified during the first week of the experiment. From day 28 to 52, NO_3^- concentrations were below the detection limit of the used kit (0.3 mg L^{-1}). After 52 days, the values oscillated between the detection limit and 21 mg kg^{-1} (figure 10).

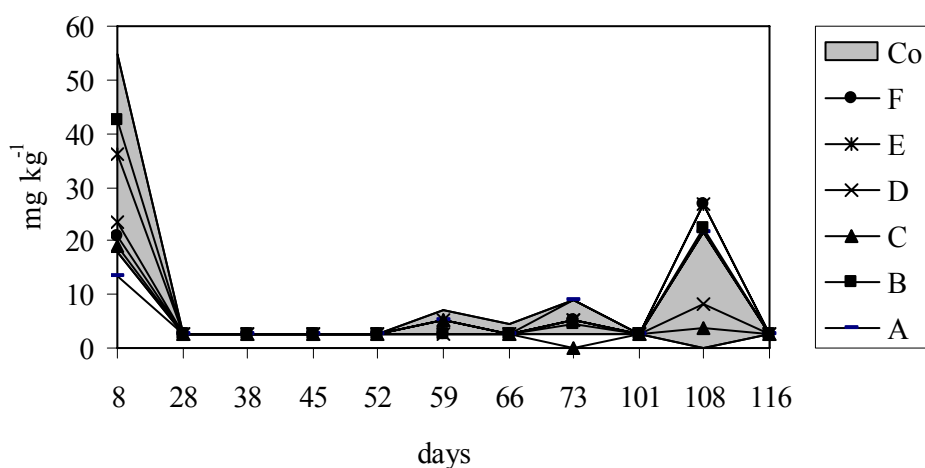


Figure 10: Course incubation measurements of NO_3^- . Cores sampled from the first cm (0 to 1) of the surface of the sediment layer of the outdoor microcosms. Co = minimum and maximum values of control microcosms, A to F = TBOH treated microcosms.

PO_4^{3-} concentration from TBOH treated microcosms was similar to the controls. Values between 4.5 and 5.3 mg kg^{-1} were detected at day 8. The PO_4^{3-} concentrations dropped to 0.4 mg kg^{-1} at day 28, but began to increase after 45 days. At day 101, the concentrations decreased to 0.4 mg kg^{-1} , but tended to rise at the end of the experiment (figure 11).

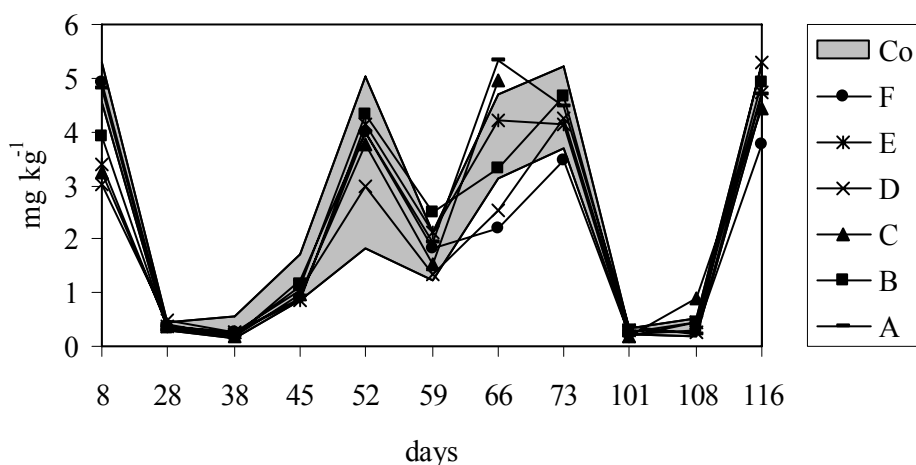


Figure 11: Course incubation measurements of PO_4^{3-} . Cores sampled from the first cm (0 to 1) of the surface of the sediment layer of the outdoor microcosms. Co = minimum and maximum values of control microcosms, A to F = TBOH treated microcosms.

The amount of NH_4^+ , NO_3^- and PO_4^{3-} quantified in the microcosms was compared with in situ conditions of the lake. No significant differences were observed for NH_4^+ and PO_4^{3-} between the microcosms and the lake ($p > 0.05$). In both cases, nitrate was below the detection limit (table 8).

Table 8: Comparison between amounts of NH_4^+ , NO_3^- , and PO_4^{3-} of the sediment surface of the Lake Ammersee and outdoor microcosms. Numbers below represent mean values of 3 measurements made on the lake at 25.07.01 and mean of values obtained for all microcosms during sampling day 52 (26.07.01). \pm = standard deviation.

	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})	PO_4^{3-} (mg kg^{-1})
Lake Ammersee	14 ± 2	Bd	6.0 ± 0.3
Microcosms	16 ± 2.3	Bd	3.6 ± 0.8

The amount of DOC was similar in all microcosms (figure 12). At the beginning of the experiment, DOC concentrations varied between 0.1 to 0.8 g kg^{-1} . Concentrations increased at sampling day 66, where the detected values were in the range of 0.6 and 1.7 g kg^{-1} . As shown for the days 101 and 108, the concentrations tended to decrease at the end of the experiment.

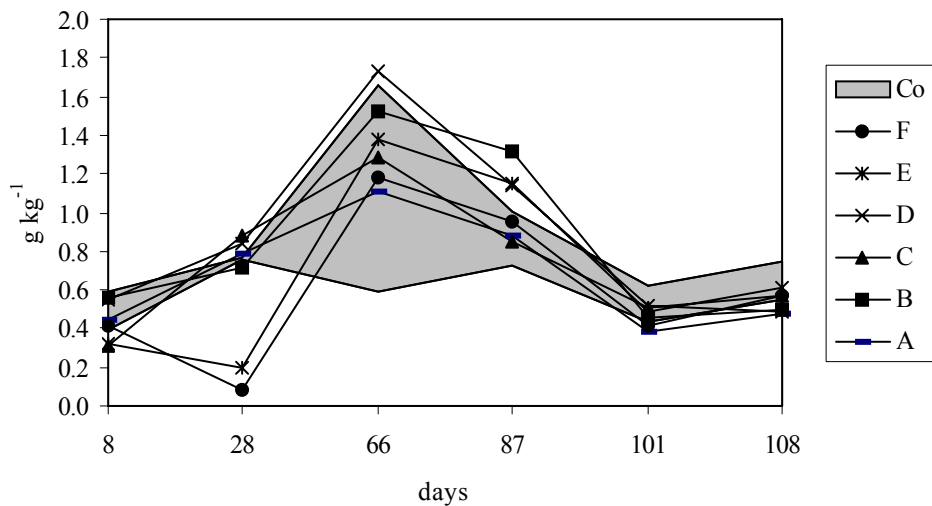


Figure 12: Course incubation measurements of DOC. Cores sampled from the first cm (0 to 1 cm) of the surface of the sediment layer of the outdoor microcosms. Co = minimum and maximum values from control microcosms. A to F = TBOH treated microcosms.

3.1.2. Structural diversity of microbial communities

3.1.2.1. Microbial diversity assessed by DGGE

3.1.2.1.1. Bacterial community structure

DGGE profiles of 16S rDNA and rRNA were used to assess TBOH-induced changes on bacterial community structure. 470 bp fragments obtained after PCR amplification using the primer set 968f GC/1401r contained the variable regions v6 to v8 of the 16S rRNA of the domain bacteria. Total community nucleic acids extracted from sediment samples (0 to 1 cm) were of high molecular weight and sufficient purity for successful amplification of the 16S rRNA and rRNA genes. In order to compare DGGE fingerprints, DICE was determinate and UPGMA was used to create dendrogram describing fingerprint similarities. Replicate samples collected from each microcosm showed similarity values of at least 93 %. This indicated a good reproducibility of nucleic acids extraction, PCR amplification and DGGE analysis. Due to the complexity of the DGGE profiles from 16S rDNA and rRNA (figure 13) only dominant bands were considered in the cluster analyses using the software Gel compar II.



Figure 13: DGGE profiles of 16S rRNA from sediment samples (0 to 1 cm) collected during the treatment phase of the outdoor microcosms experiment. Lanes: Co 4 = control microcosm 4, F = TBOH treated microcosm F and M = 100 bp molecular weight marker.

Figure 14 shows dendrograms constructed from 16S rDNA fingerprints of samples collected during pre-treatment, treatment and post-treatment phases. Before TBOH treatment, the microcosms were more than 97 % similar. During the treatment phase TBOH-induced shifts were not detected as the profiles of control and TBOH treated samples remained at least 94% similar. The same tendency was observed for the post-treatment phase.

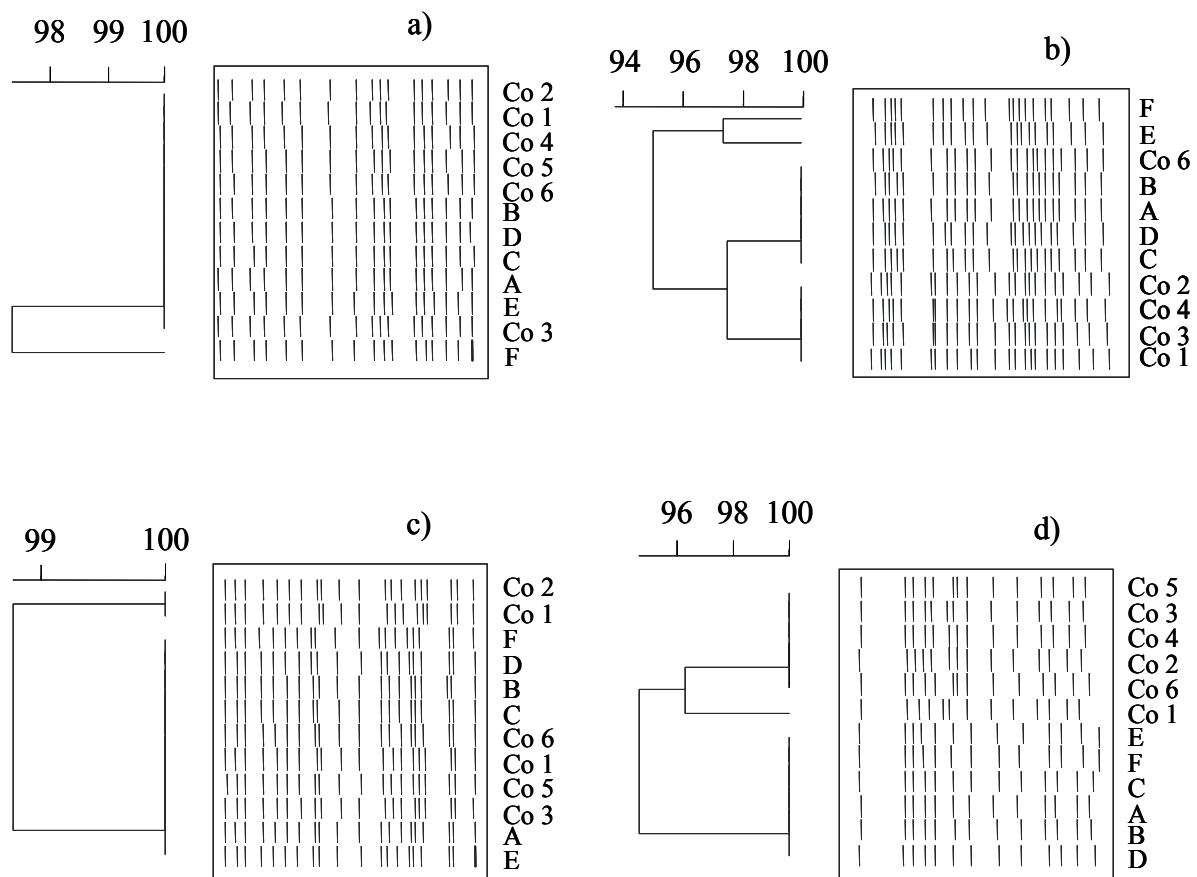


Figure 14: Dendrogram constructed from 16S rDNA fingerprints using universal primers for bacteria. Sediment samples collected during the pre-treatment (a), treatment (b and c) and post-treatment phases (d) of the outdoor microcosm experiment. Co 1-Co 6 = replicates of control microcosms; A-F = TBOH treated microcosms. The scales represent the percentage of similarity between the samples.

16S rRNA analyses were conducted with sediment samples (0 to 1 cm) collected from the control microcosm Co 4 and treated microcosms A, C and F (figure 15). Before TBOH treatment, fingerprints from all microcosms were at least 92% similar. During the treatment and post-treatment phases, the similarity between control and TBOH treated microcosms increased to a minimum of 97%. These results showed that TBOH-induced shifts on the profiles did not occur, corroborating DNA-based analysis.

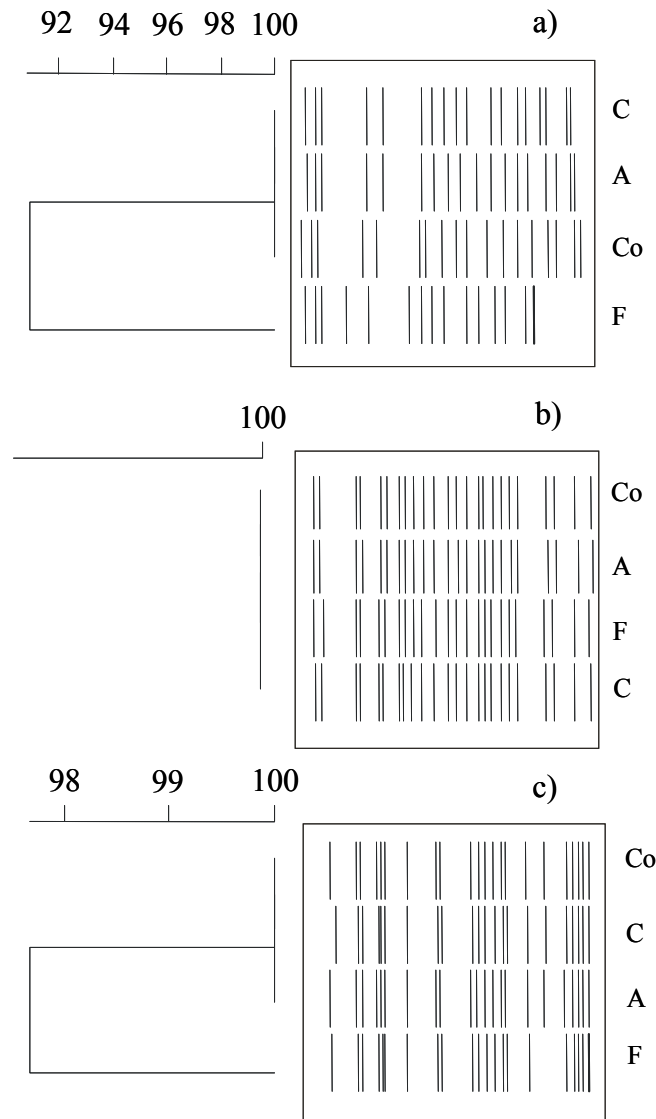


Figure 15: Dendrogram constructed from 16S rRNA fingerprints using universal primers for bacteria. Sediment samples collected during the pre-treatment (a), treatment (b) and post-treatment phases (c) of the outdoor microcosm experiment. Co = control microcosm 4, A, C and F = TBOH treated microcosms A, C and F, respectively. The scales represent the percentage of similarity between the samples.

Samples from all phases of the experiment were compared in a single DGGE gel to study the development of the bacterial communities in the microcosms. The high similarity (more than 95 %) between samples taken at different phases of the experiment (figure 16) indicated that the bacterial community structure remained very stable along the experiment.

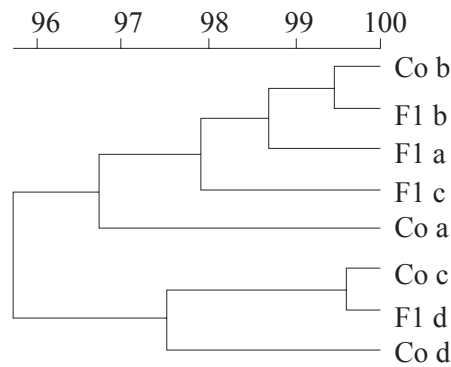


Figure 16: Comparison between 16S rDNA fingerprints using universal primers for bacteria of samples collected during the pre-treatment (a), treatment (b and c) and post-treatment (d) phases of the outdoor microcosm experiment. Co = control microcosm 4. F = TBOH treated microcosm F. The scale represents the percentage of similarity between the samples.

Figure 17 shows the comparison between fingerprints of samples collected from the microcosms A and the surface sediment (0-20) of the Lake Ammersee. Samples were taken at the same week during the treatment phase. As the microcosms fingerprints were very similar, only control microcosm 4 was used for the comparison. Dominant bacterial populations from the microcosms were nearly identical to the one found in the lake, with similarity coefficients higher than 99%.

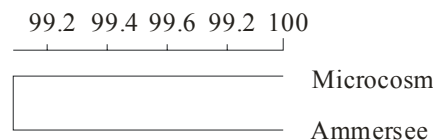


Figure 17: Comparison between 16S rDNA fingerprints of the sediment from the lake Ammersee and the microcosm Co 4. Samples taken at the same week during the treatment phase. The scale represents the percentage of similarity between the samples.

3.1.2.1.2. Archaeal community structure

The primer set 039W/036W was used to amplify a fragment of the 16S rRNA genes of archaeal communities of the control microcosm Co 4 and TBOH treated microcosms A, C and F. As the relationship between rRNA content and activity has been less investigated for archaea than for bacteria, 16S rRNA analyses of 16S rRNA were not conducted for this domain. PCR products of correct size could be obtained from all analyzed sediment samples. The triplicate samples of each microcosm showed at least 82 % similarity. Five to 9 bands could be detected in the DGGE gels. Fingerprints of samples collected during the pre- and post-treatment phases showed more than 93% similarity between control and TBOH treated microcosms (figure 18). In spite of the lower percentage of similarity detected for the treatment phase, no clear effects of TBOH were observed.

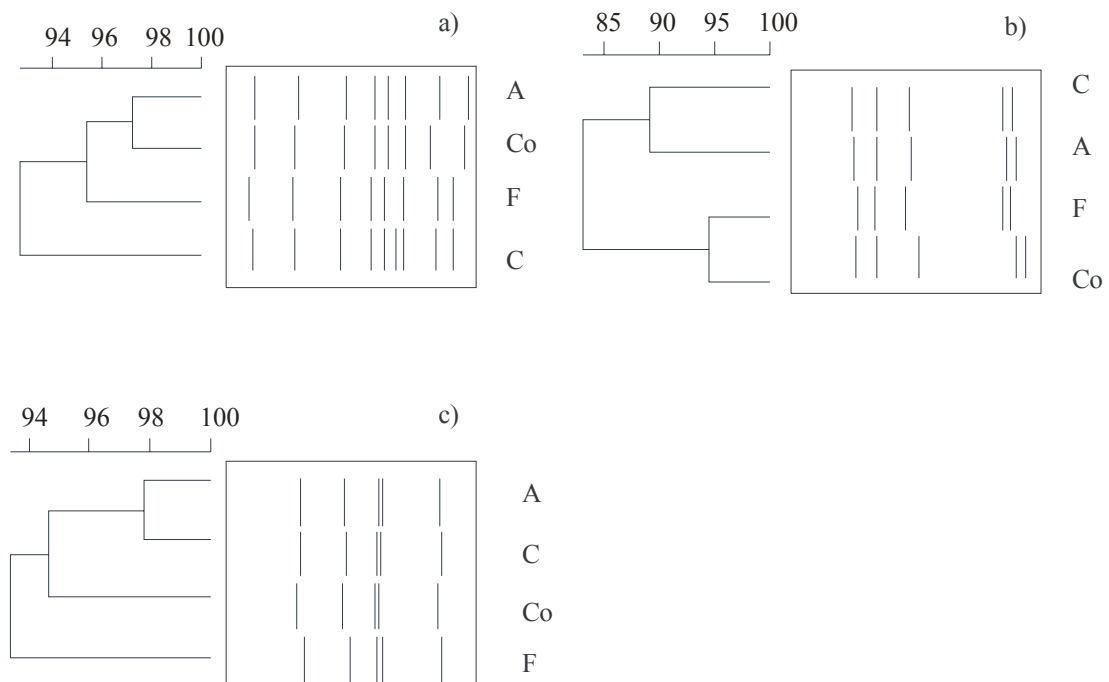


Figure 18: Dendrogram constructed from the 16S rDNA fingerprints using primers for archaea. Sediment samples (0 to 1 cm) collected during the pre-treatment (a), treatment (b) and post-treatment phase (c) of the outdoor microcosm experiment. Co = microcosm 4; A, C and F = TBOH treated microcosms A, C and F, respectively. The scale represents the percentage of similarity between the samples.

3.1.2.2. Bacterial diversity by 16S rDNA clone libraries

Since the DGGE analysis showed high similarity between 16S rRNA and rDNA fingerprints clone libraries were constructed using only 16S PCR products of DNA extracts from microcosms Co 4 and F, chosen as representative samples of control and TBOH treated sediment. 61 and 113 clones were obtained, respectively, in control and treated sediment libraries. However, only 52 and 80 clones contained inserts of the correct size. 52 clones from the control library and 54 clones representing different RFLP patterns found in the treated sediment library were submitted to phylogenetic analyses.

Rarefaction's curves were made for each clone library. In these curves the number of different bacterial groups is plotted versus the number of analyzed clones, to determine if the number of analyzed clones was satisfactory to cover bacterial diversity in a certain environment. As shown in figure 19, a plateau was not be fully reached for both curves indicating that more than 50 clone should be analyzed to get more precise information about the bacterial diversity in this sediment. However, a detailed investigation on the bacterial diversity of these sediments was out of the scope of the present work.

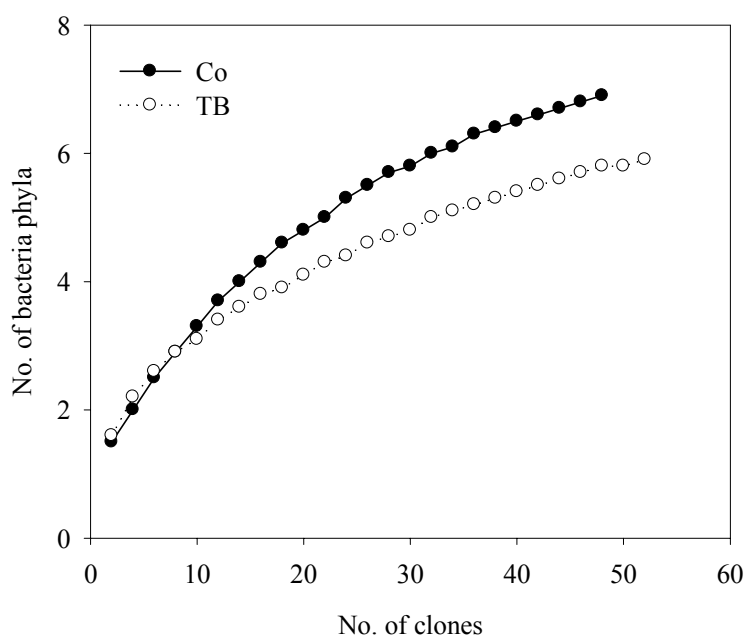


Figure 19: Rarefaction's curves showing the number of different bacterial phyla plotted as a function of number of clones. Co = Control sediment 16S rDNA clone library, TB = TBOH treated sediment 16S rDNA clone library.

Seven different bacterial phyla were detected for both clone libraries. The distribution of clones among the phyla was uneven. As shown in figure 20, 72 and 62 % of sequences detected for control and treated sediment 16S rDNA clone libraries belonged to the *Proteobacteria*. TBOH treated differed from the untreated sediment in non-dominant groups. The phyla *Verrucomicrobia*, *Firmicutes* and *Chlorobi* were detected in control but not in the treated sediment library. On the other hand, *Chloroflexi*, *Cyanobacteria* and *Planctomycetes* were only present in the treated sediment library.

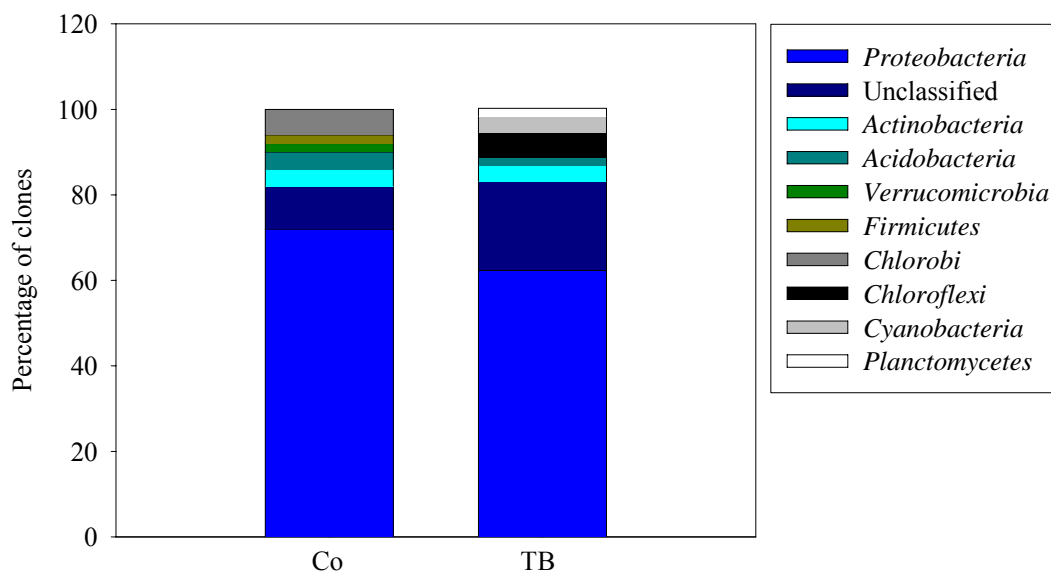


Figure 20: Distribution of clones in the different bacterial phyla. Control (Co) and TBOH treated (TB) = 16S rDNA clone libraries generated from samples collected during the treatment phase from microcosm Co4 and F, respectively.

3.1.2.2.1. Division *Proteobacteria*

Since most clones found in both clone libraries belonged to the *Proteobacteria*, this phylum was analyzed in more detail. Clones of the β -class present in the control and treated sediment libraries represented 78 and 58 % of the *Proteobacteria*, respectively (figure 21). The remaining clones of the control library were distributed among the γ - and α -classes, accounting for 13 and 9 % of the *Proteobacteria*. In the treated sediment library, the γ - and α -classes represented 8 and 35 % of the *Proteobacteria*.

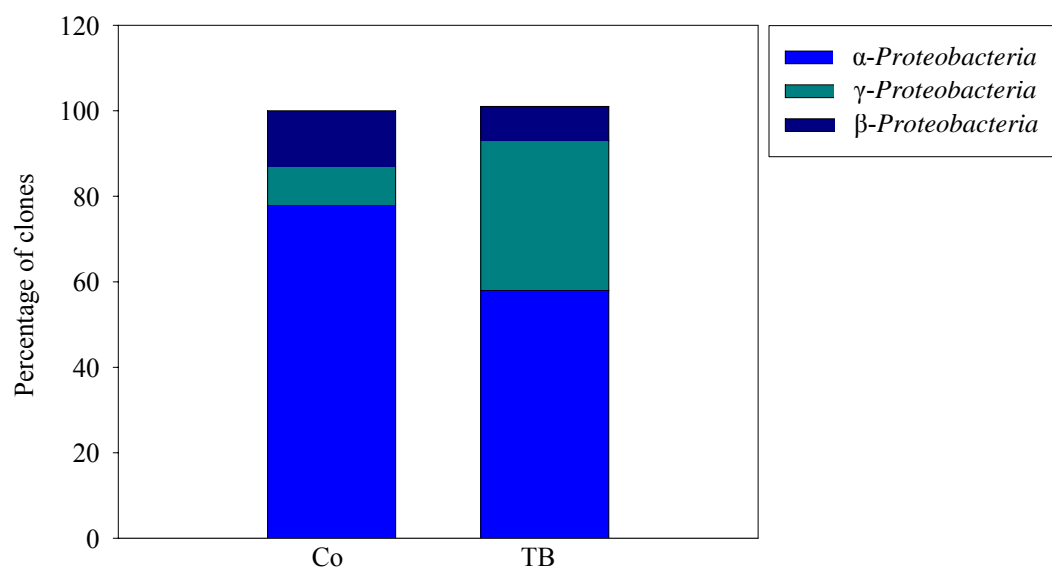


Figure 21: Distribution of the clones in the *Proteobacteria* phylum. Control (Co) and TBOH treated (TB) = 16S rDNA clone libraries generated from samples collected at the treatment phase from microcosm Co4 and F, respectively.

Distinct clusters representing the majority of the clones of the Proteobacteria phylum were observed for each library (Figure 22). In the control library, 11 clones clustered with sequences from the *Burkholderia* genus. In the TBOH treated sediment library, 5 clones formed a cluster with the sequence of an ammonia-oxidizing bacterium (*Nitrosococcus* sp.). The remaining clones were distributed throughout the phylogenetic tree.

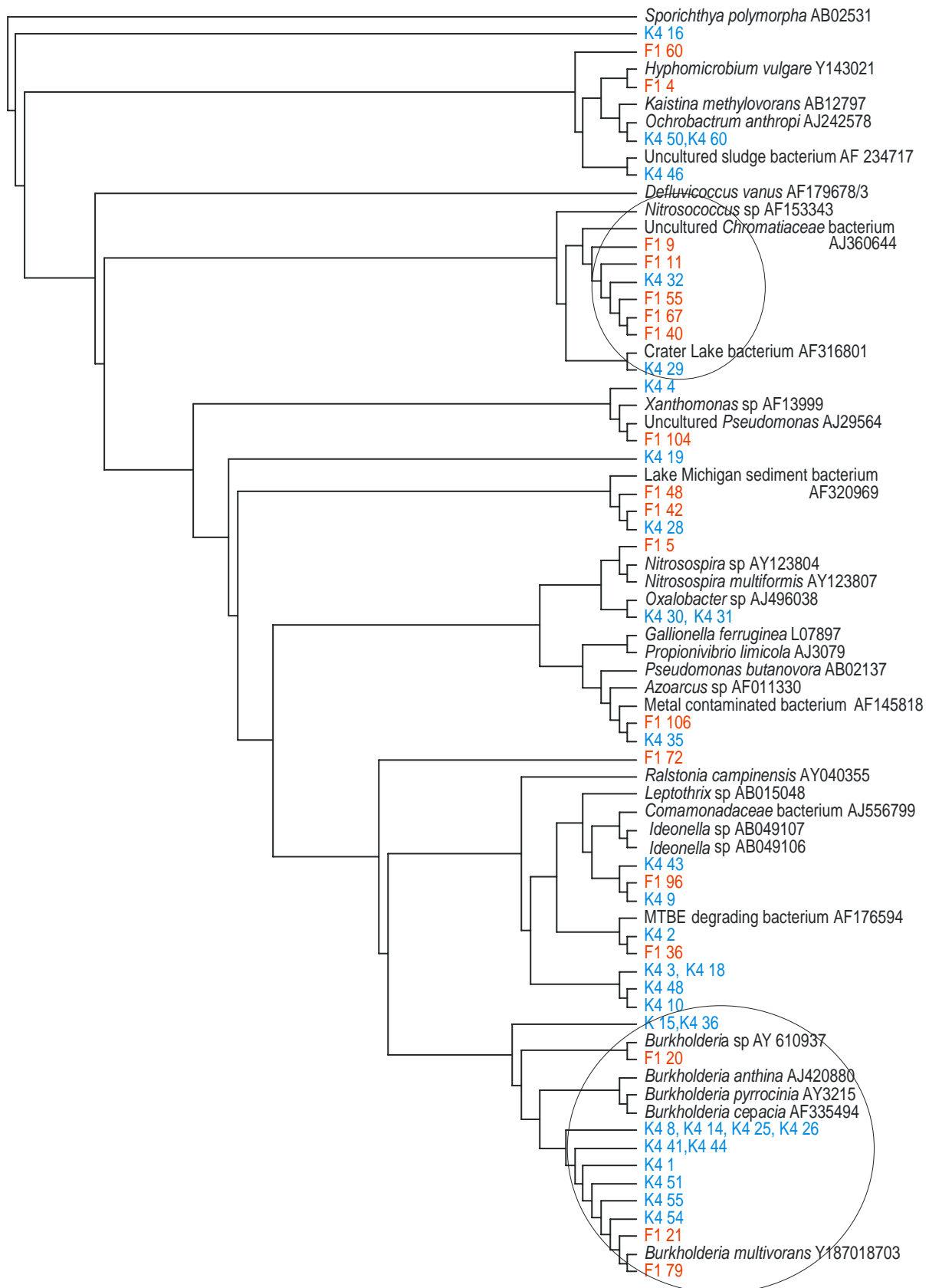


Figure 22: Phylogram showing the relationship of clones obtained from sediment samples and database-sequences of the *Proteobacteria* phylum. K4 = clones from control microcosm 4 and F = clones from TBOH treated microcosm F; Circles = clusters representing the majority of the clones from the *Proteobacteria* phylum. *S. polymorpha* AB02531 was used as out-group.

3.2. Effects of TBOH: laboratory microcosm experiment

3.2.1. Abiotic parameters

Samples collected from the surface (0 to 5 cm) of the sediment layer at the end of the microcosm incubation were used to quantify DOC, NH_4^+ , PO_4^{3-} and NO_3^- (table 9). The amount of DOC and NH_4^+ did not significantly differ ($p > 0.05$) in the sediment samples of control and the treated microcosms. The values were in the range of 1.17 to 1.28 g of DOC per kg of dry sediment, and 11.52 to 20.19 mg of NH_4^+ mg kg^{-1} . The PO_4^{3-} concentration was lower in TBOH treated microcosms compared to control microcosms (0.61 vs. 1.1 mg kg^{-1}), although this effect did not prove to be statistically significant. The values of NO_3^- were below the detection limit of the kit (0.3 mg L^{-1}).

Table 9: Amount of DOC, NH_4^+ , PO_4^{3-} in the surface (0 to 5 cm) of the sediment layer of the laboratory microcosms. Co 1-4 = replicates of control microcosms; TB 1-4 = replicates of TBOH treated microcosms; \pm = standard deviation.

	NH_4^+ (mg kg^{-1})	Ortho- PO_4^{3-} (mg kg^{-1})	DOC (g kg^{-1})
CO 1	20.19 \pm 3.96	1.02 \pm 0.33	1.28 \pm 0.04
CO 2	15.87 \pm 2.33	0.82 \pm 0.09	1.27 \pm 0.02
CO 3	17.66 \pm 3.85	1.21 \pm 1.06	1.17 \pm 0.01
CO 4	18.14 \pm 1.1	0.63 \pm 0.1	1.27 \pm 0.02
TB 1	16.96 \pm 5.26	0.88 \pm 0.08	1.26 \pm 0.04
TB 2	13.92 \pm 1.49	1.1 \pm 0.2	1.23 \pm 0.02
TB 3	11.52 \pm 3.98	0.64 \pm 0.07	1.21 \pm 0.08
TB 4	16.14 \pm 2.49	0.61 \pm 0.21	1.21 \pm 0.02

3.2.2. Structural diversity of microbial communities

3.2.2.1. Total cell counts

Cells extracted from sediment were quantified by epifluorescence microscopy, using DAPI staining (figure 23). Total cell counts showed no significant differences between microcosm replicates as well as between control and treated microcosms ($p > 0.05$; 1.7 and 2.1 $\times 10^8$ cells g^{-1} , respectively).

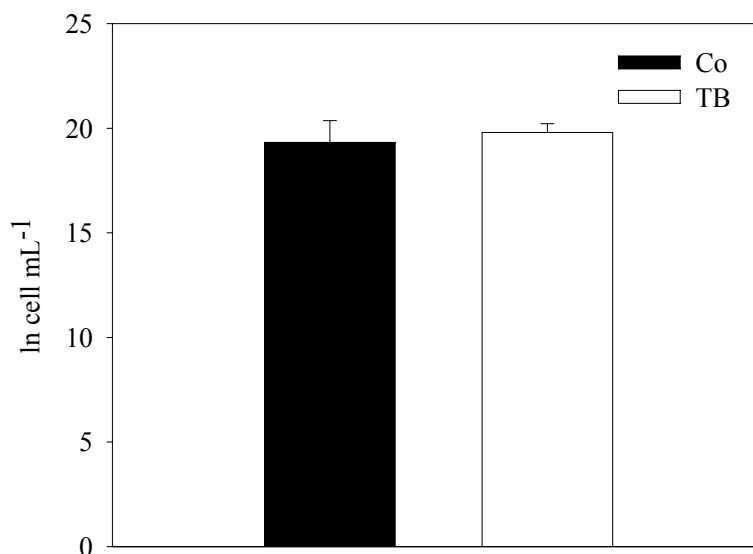


Figure 23: Total cell counts from cell suspensions obtained from sediment samples from the laboratory microcosm experiment, using DAPI staining. Co = mean values of control microcosms, TB = mean values TBOH treated microcosms.

3.2.2.2. Total genetic diversity assessed by RAPD

RAPD analysis was used to assess changes in the gene pool directly extracted from the sediment. PCR product size varied from 250 bp to 2000 bp (figure 24). Replicates showed more than 99 % similarity, indicating a high reproducibility of the method. Before TBOH treatment, fingerprints from replicate microcosms were almost identical (99 % similarity) (figure 25c). 10 days after TBOH application, replicates of control and treated microcosms formed clusters, which were 4 % different from each other (figure 25a). Similar results were observed 14 days after TBOH application (figure 25b). However, for this sampling data the microcosm Co 2 grouped with treated microcosms (100% similarity) (figure 25b). The remaining control microcosms formed a cluster with 97 % similarity. 19 days after TBOH treatment, the similarity coefficient of replicates of control as well as treated microcosms was slightly reduced (92 % similarity) (figure 25c). Microcosm TB 4 was the only exception and showed a different development of the total gene pool with only 82 % similarity to all other microcosms analyzed at the end of the experiment. Differences were found when comparing fingerprints before and 19 days after TBOH treatment (similarity values < 70 %), indicating a shift in the gene pool over the experiment in all microcosms. However, no clear TBOH-induced changes were observed, as control and treated microcosms showed at least 92 % similarity in all sampling dates (10, 14 and 19 days after TBOH application).

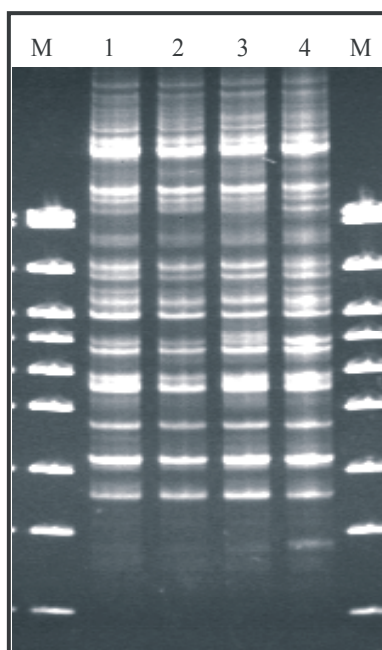


Figure 24: RAPD fingerprints of sediment samples (0 to 1 cm) collected during the treatment phase of the laboratory microcosm experiment. Lanes: 1-4 = replicates of control microcosm 4, 100 bp molecular weight marker.

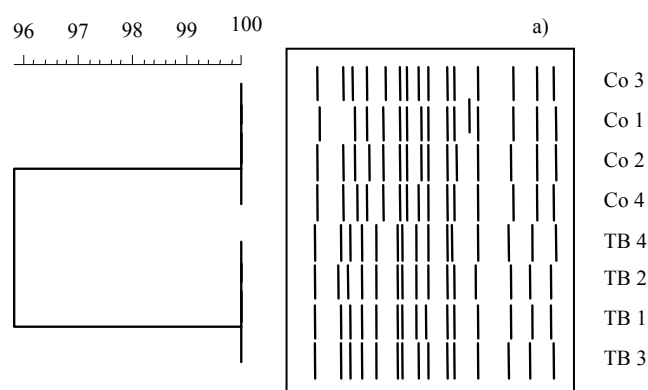


Figure 25: Dendrogram constructed from RAPD fingerprints of sediment samples. a) samples collected 10 days after TBOH treatment, b) samples collected 14 days after TBOH treatment and c) comparison between the RAPD fingerprints before (a) and 19 days after (b) TBOH treatment. Co 1-4 = replicates of control microcosms and TB 1-4 = replicates of TBOH treated microcosms. The scales represent the percentage of similarity between the samples. To be continued.

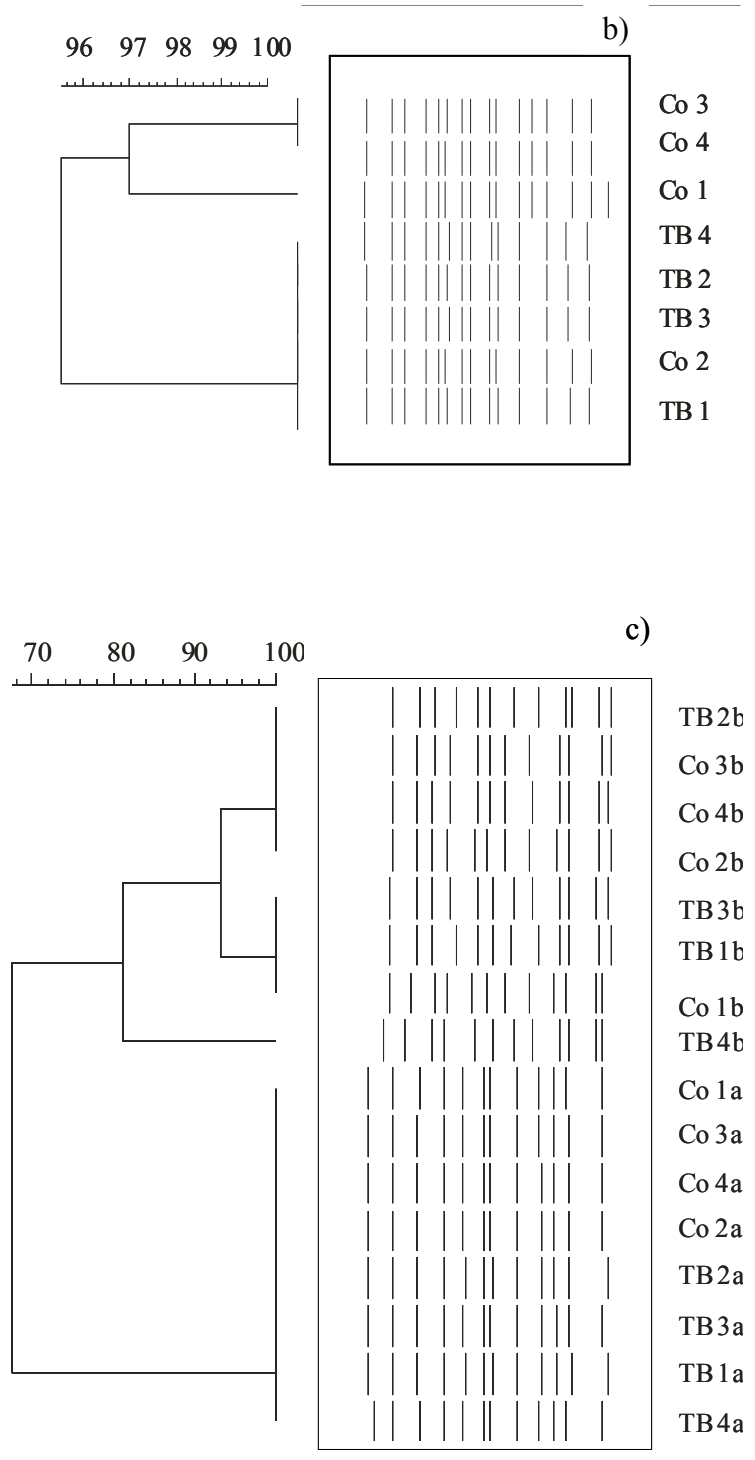


Figure 25: continued.

3.2.2.3. Bacterial community structure by 16S rRNA/rDNA-DGGE

As showed in figure 26, the DGGE profiles of 16S rRNA and rDNA directly extracted from the sediment were highly complex. For rDNA as well as for rRNA fingerprints the method was reproducible, with similarity values of more than 98 % between replicate extracts of the same sample. The similarity between the profiles of replicates of treated as well as control microcosms was higher than 93 %. Cluster analysis did not indicate a consistent variation in the profiles which could be attributed to TBOH treatment (figure 27). Profiles of rRNA and rDNA simultaneously extracted from sediment samples were at least 90.2 % similar to each other.

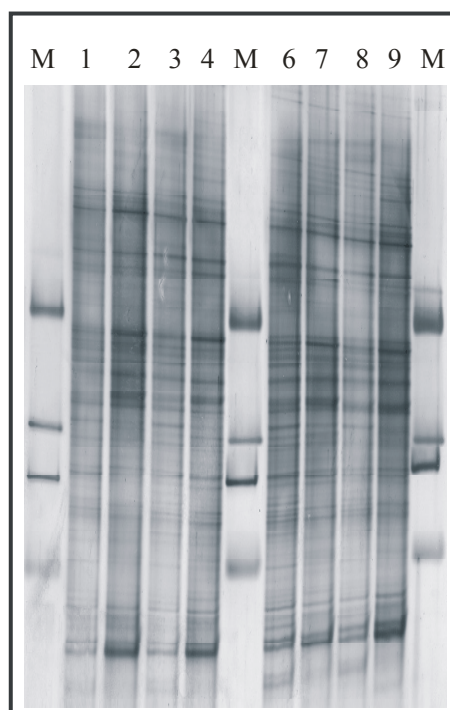


Figure 26: DGGE profiles of 16S rRNA and rDNA amplicons from sediment samples collected 19 days after TBOH treatment (laboratory microcosms experiment). Lanes: M = 100 bp molecular weight marker; 1 and 3 = rDNA from control microcosms 1 and 2, respectively; 2 and 4 = rRNA from control microcosms 1 and 2, respectively; 6 and 8 = rDNA from TBOH treated microcosms 1 and 2, respectively, 7 and 9 = rRNA from TBOH treated microcosms 1 and 2, respectively.

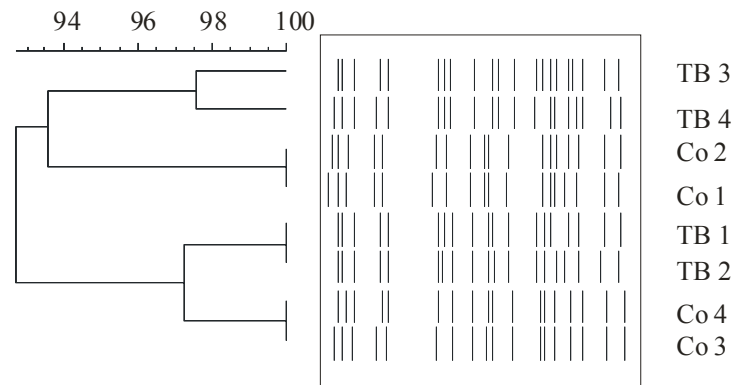


Figure 27: Dendrogram constructed from 16S rRNA fingerprints using universal primers for bacteria. Sediment samples collected 19 days after TBOH treatment (laboratory microcosms experiment). Co 1-4 = replicates of control microcosms and TB 1-4 = replicate of TBOH treated microcosms. The scale represents the percentage of similarity between the samples.

3.2.3. Microbial community function

3.2.3.1 Genes involved in carbon and nitrogen turnover

Effects of TBOH on microbes involved in carbon and nitrogen turnover were assessed by the detection of presence and expression of nitrite reductase (*nir S* and *nir k*) and chitinase (subfamily 18 group A) genes. Sequences obtained from a clone of a sediment sample proofed the specificity of the used primers. Three replicate samples were analyzed per microcosm. In both, control and TBOH treated microcosms only *nir S* genes could be found in sediment samples. However, an expression of these genes could not be detected. Similar results were observed for chitinase genes. Moreover, the analysis of DGGE profiles of chitinase genes found in sediments samples showed that 5 different genes could be detected by the used primers (figure 28). However, no differences were observed between profiles of chitinase genes from control and treated microcosms (100% similarity). The PCR product size (890 bp) obtained by amplification with *nir S* primers did not allow further DGGE analysis.

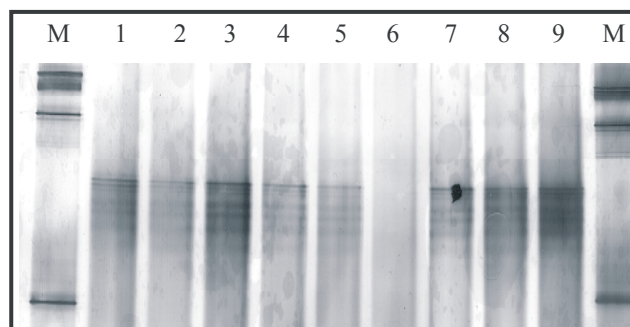


Figure 28: DGGE profiles of group A bacterial chitinase genes. Lanes: M = 100 bp molecular weight marker; 1-3 = control microcosms; 4-6 = TBOH treated microcosms 1; 7-9 = TBOH treated microcosm 2.

3.2.3.2. Enzyme activities

Effects of TBOH treatment on microbial community functions were investigated using enzyme micro titer plate assays. Figure 29 shows the activity of the enzymes phosphomonoesterase, leucine-aminopeptidase, β -glucosidase and N-acetyl-glucosaminidase measured from filtered sediment suspensions collected 19 days after the addition of TBOH. The median values of β -glucosidase and leucine-aminopeptidase activities were slightly lower in samples from TBOH treated microcosms. For phosphomonoesterase, the activity was lower in the control. A significant effect was only observed for N-acetyl-glucosaminidase ($p < 0.01$), in which the activity from treated samples 50% lower than the controls.

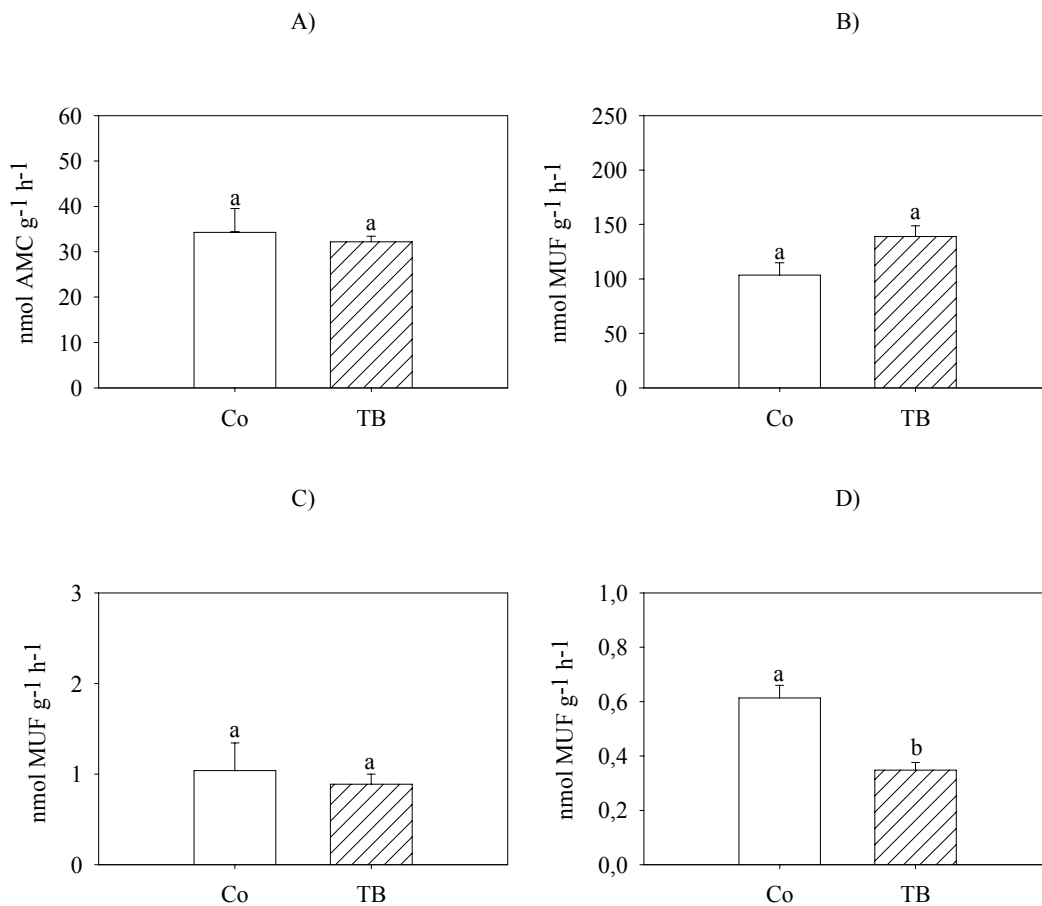


Figure 29: Enzyme activities of sediment samples taken 19 days after TBOH application (laboratory microcosm experiment). A) Leucine-aminopeptidase; B) phosphomonoesterase; C) β -glucosidase; D) N-acetyl-glucosaminidase. Co = control microcosms and TB = TBOH treated microcosms. a = $p > 0.05$; a and b = $p < 0.05$.

3.2.3.3. Substrate utilization potential

After 19 days of treatment, communities from both control and treated microcosms were assessed for their tolerance towards TBOH using BIOLOG[®] GN plates. Cells extracted from control and TBOH treated sediment were inoculated in micro titer plates containing 95 different carbon sources and with or without 100 ng L⁻¹ of TBOH. Microbial communities extracted from control and TBOH treated microcosm are referred here as control and treated communities. Figure 30 shows the number of positive wells obtained during a 184 h incubation period. Measurements were done at 40, 88 and 184 h. Characteristic sigmoid responses were observed, with substrate utilization detected at higher rates in plates inoculated with microbial communities from control microcosms without any further treatment (CO). After 40 h of incubation, the number of positive wells was higher in plates inoculated with control communities without any TBOH addition (CO = 23.5) than in plates containing control communities and 100 ng mL⁻¹ of TBOH (COTB = 14.5). For the treated communities, plates without additional TBOH treatment (TB) and plates containing 100 ng mL⁻¹ of TBOH (TBTB) had very similar values, 14 and 14.5, respectively. After 88 and 184 h, the positive wells increased in all plates, but differences between treatments were maintained.

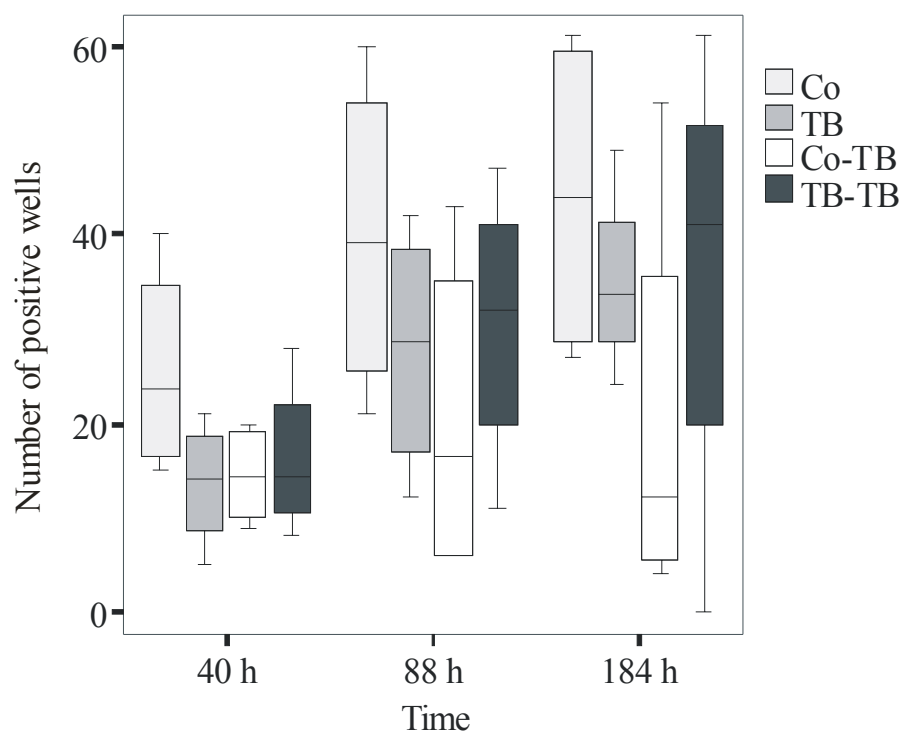


Figure 30: Substrate utilization potential of microbial communities from sediment samples measured using BIOLOG GN plates without and with 100 ng mL^{-1} TBOH (laboratory microcosm experiment). Samples were taken 19 days after TBOH application. Cells were extracted from control and treated sediments and suspensions of $10^6 \text{ cells mL}^{-1}$ were applied in the plates. CO = microbial community from control sediment; COTB = microbial community from control sediment treated with 100 ng mL^{-1} TBOH; TB = microbial community from TBOH treated sediment; TBTB = microbial community from TBOH treated with 100 ng mL^{-1} TBOH.

3.3. Overall microbial activity and biomass in the sediment by microcalorimetry

The overall activity from sediment samples collected from the lake Ammersee was determined by microcalorimetry. Independent measurements showed the same microbial activity values, indicating the reproducibility of the method. The basal activity of the sediment, which is defined as the activity quantified without any substrate addition, started with values of $50 \mu\text{W}$ per gram of dry sediment and slowly decreased with time (figure 31). Samples enriched with Lake Ammersee sediment DOC showed the same activity as the basal (figure 31). The addition of 4 mg of yeast extract per g of dry sediment increased the total activity up to 160% (data not shown). As shown in figure 32, TBOH treatment ($15 \mu\text{g kg}^{-1}$) caused a light reduction of the total microbial activity.

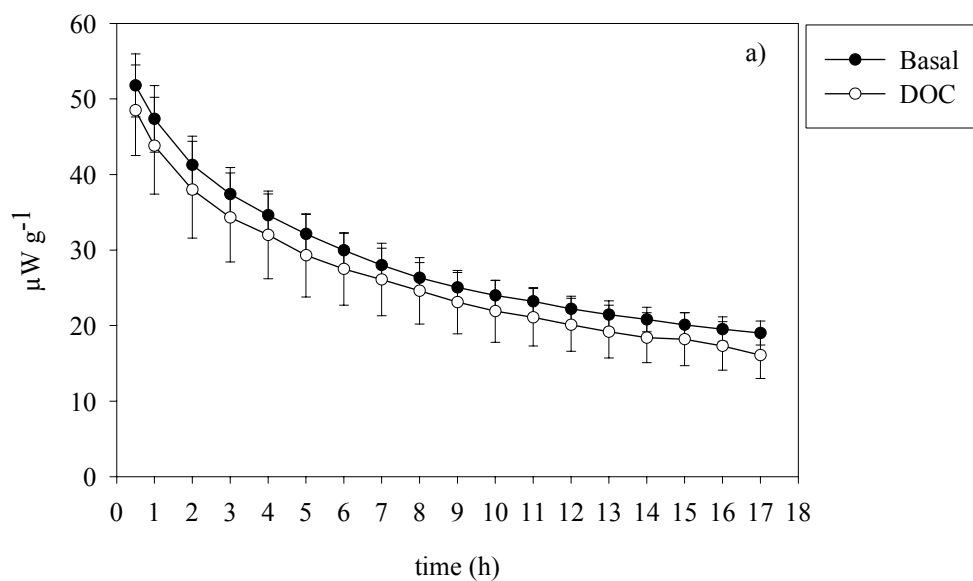


Figure 31: Microbial activity measured from sediment samples by microcalorimetry. Basal = sediment without any treatment; DOC = samples supplied with Lake Ammersee DOC.

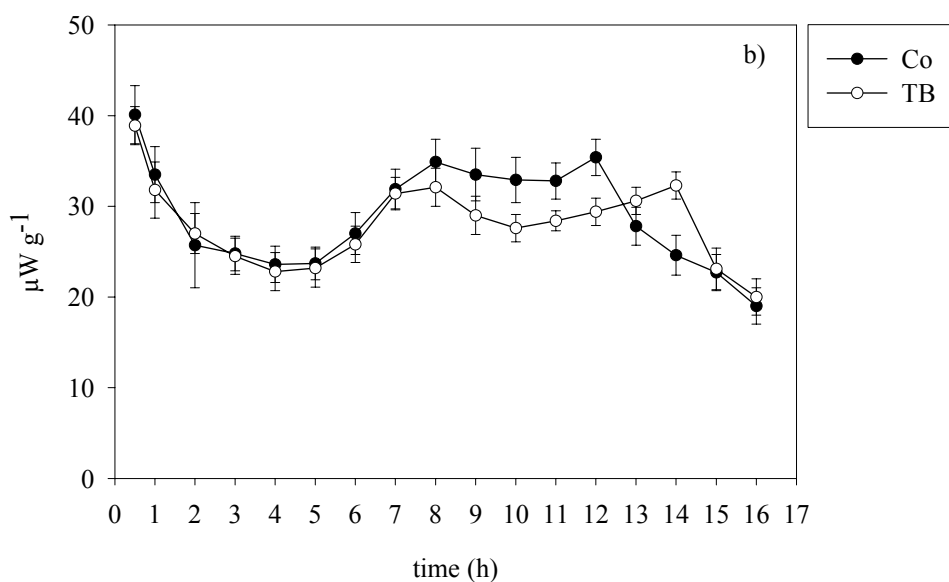


Figure 32: Microbial activity measured from sediment samples by microcalorimetry. Co = sediment supplied with 4 mg g⁻¹ yeast extract; TB = sediment samples supplied with 4 mg g⁻¹ yeast extract and 15 $\mu\text{g kg}^{-1}$ TBOH. Plot shows the difference between the activity total quantified and the basal activity.

Effects of TBOH on active microbial biomass were calculated on basis of microcalorimetry measurements using the equation developed by Sparling (1983), as given in 2.5.3. Microbial biomass was identical for sediment samples incubated with 4 mg g⁻¹ yeast extract and without or with 15 µg kg⁻¹ TBOH (figure 33).

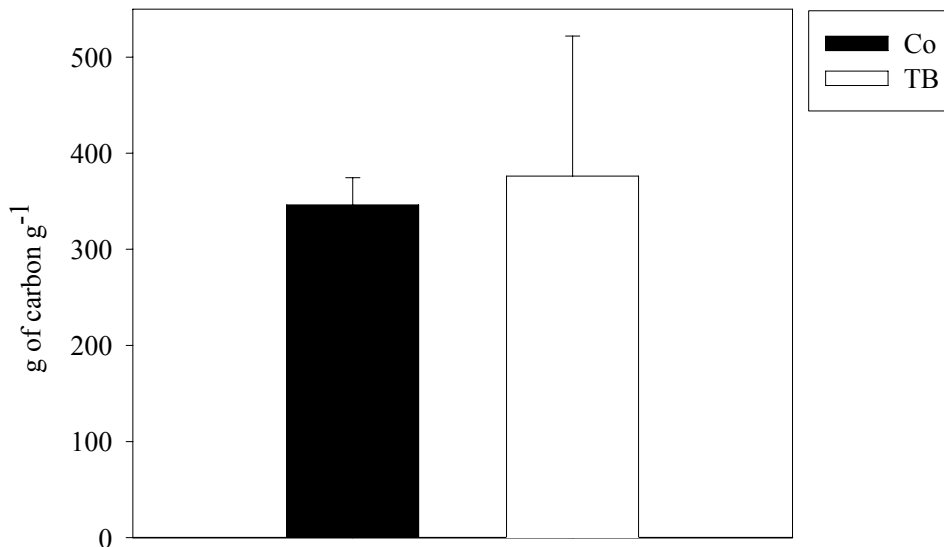


Figure 33: Microbial biomass from sediment samples supplied with 4 mg g⁻¹ yeast extract and without (Co) or with 15 µg kg⁻¹ TBOH (TB).

3.4. Effects of TBOH on the structure of metabolic active bacterial populations

The bacterial strains *Azospirillum brasiliense* Sp 7 (DSMZ 1690), *Burkholderia cepacia* (DSMZ 7288), *Pseudomonas fluorescens* (DSMZ 50090), *Arthrobacter citreus* BI 90, *Cytophaga* sp (DSMZ 3661) and *Bacillus subtilis* (DSMZ 10) were chosen as representatives of major bacterial groups to test their ability to incorporate BrdU. Only *B. cepacia*, *A. citreus* and *Cytophaga* sp could incorporate BrdU, in pure culture, by the used experimental condition.

DNA and RNA extracted from sediment samples incubated with BrdU were used, respectively, for the BrdU-immunocapture and 16S RT-PCR. The immunocapture allowed the separation of BrdU-labeled DNA (beads bound fraction) from the non-labeled DNA (supernatant). This was proofed by the absence of PCR products using DNA templates (beads

bound fraction) of sediment samples incubated without BrdU. No 16S rDNA PCR products were detected for DNA of sediment samples (beads bound fraction) incubated with BrdU without nutrient supply. However, after addition of 4 mg g^{-1} yeast extract 16S rDNA could be amplified from both control and TBOH treated sediment samples.

DGGE fingerprints were generated from the 16S rDNA from labeled and non-labeled DNA as well as 16S rRNA (figure 34). 16S fingerprints obtained from the rRNA and non-labeled DNA were very similar to each other and more complex than fingerprint from the labeled DNA, indicating a lower diversity of metabolic active organisms that took up BrdU. Although the same bands were present in fingerprints of BrdU-labeled DNA from control and TBOH treated samples, the intensity of some bands were shown to be different (figure 34, arrows).

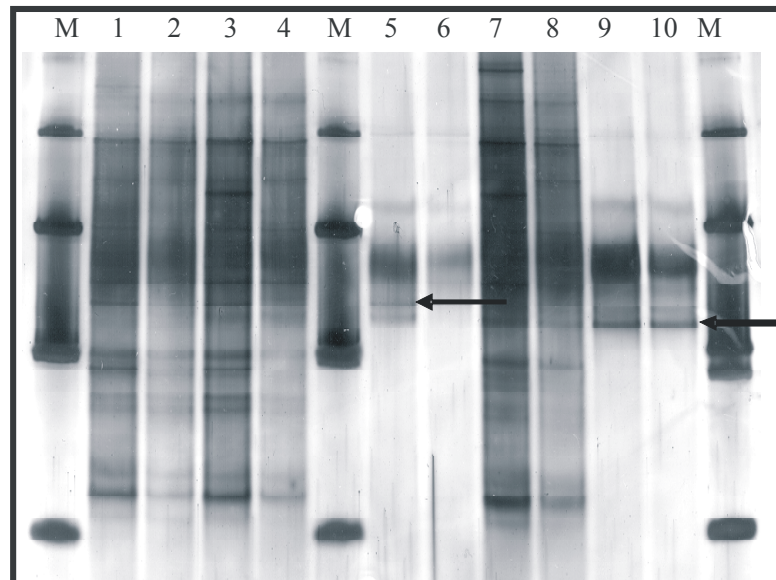


Figure 34: DGGE profiles of 16S rDNA from BrdU-labeled and non-labeled DNA as well as 16S rRNA. Lanes: M = 100 bp molecular weight marker; 1 = 16S rDNA from control sediment (without any treatment); 2 = 16S rRNA from control sediment; 3 = 16S rRNA from sediment samples amended with yeast extract and BrdU; 4 = 16S rDNA of non-labeled (immunocapture supernatant) from sediment samples amended with yeast extract and BrdU; 5 and 6 = 16S rDNA of labeled DNA from sediment samples amended with yeast extract and BrdU; 7 = 16S rRNA from sediment samples amended with 0.5 mg g^{-1} TBOH, yeast extract and BrdU; 8 = 16S rDNA of non-labeled DNA from sediment samples amended with 0.5 mg g^{-1} TBOH, yeast extract and BrdU; 9 and 10 = 16S rDNA of labeled DNA from sediment samples amended with 0.5 mg g^{-1} TBOH, yeast extract and BrdU. Black arrows = bands with different intensity in control and TBOH treated samples.

4. Discussion

In the last decades scientists worldwide have demonstrated changes in the wild life, mainly in aquatic ecosystems, caused by substances that interfere with the endocrine system. Although many studies concerning this problem are helping to clarify the origin of EDCs and their effects on vertebrates, possible effects of these substances on non-target organisms, especially microorganisms, have been little investigated. Investigation of benthic microbial community should be considered of particular importance as sediment microbiota not only mediate biogeochemical transformations of global significance but are essential to the maintenance of clean water, organic matter decomposition, mineral recycling, primary production and the uptake and transfer of materials (including contaminants). Moreover, sediments were shown to be major sink and potential source of persistent EDCs. Therefore, the present study investigated the effects of the androgen TBOH on the structure and functions of microbial community from the Lake Ammersee sediment. The study was performed at different scales, in outdoor and laboratory microcosm systems. The outdoor microcosm experiment allowed the investigation of long-term effects of TBOH, continuously released into the water, on benthic microbial communities, taken into account possible interactions with other trophic levels. In the laboratory experiment, short-term effects on microbial communities, caused by a single application of TBOH, were investigated. The results might be interesting not only for ecologists but also for industry, politics and society as most of these chemicals accumulate since decades in our lakes and rivers.

4.1. Effects of TBOH on cell density and biomass

Neither toxic nor stimulatory effects were caused by TBOH treatment on total cell counts, as 10^8 cells per gram of sediment were detected in control and treated microcosms by direct count. Jontofsohn et al (2002b) found similar numbers in a analogous microcosm experiment, ran during the year of 1998. However, the application of the EDC nonylphenol caused a dose-dependent increase of microbial numbers. In the present study, the applied TBOH concentration, which was around $15 \mu\text{g kg}^{-1}$ considering the whole microcosm volume, was probably too low to stimulate microbial growth, serving as carbon source, or to inhibit cell division and/or cause cell death. A toxicity study done with *Salmonella typhimurium* showed that $111 \mu\text{g} (\pm 5.5 \mu\text{g mL}^{-1})$ of TBOH reduced 50 % of its growth on agar plates (Lutz et al., 1988). This may not perfectly reflect the effects of EDCs on microbial communities, as those

tests are normally made under ideal growth conditions which do not occur in the environment. However, it indicates that higher pollutant may be required to directly affect microorganism. Moreover, bias on investigations on effects of pollutants on cell density using direct counts methods may occur, as non-bacterial particles can also cause fluorescence (Novitsky, 1987) and cells are quantified independently of their metabolic state or viability. As showed by Girvan et al (2004), in agricultural soils, pesticide treatment negatively affected the cultivable fraction of bacterial community without changing total cell counts. Especially in the case of sediments, which are known to contain high percentage of metabolic inactive cells (Luna et al., 2002), this problem must be taken into account.

Nevertheless, microbial biomass results corroborate the cell counts, as no differences were observed between control and TBOH treated samples. Widenfalk et al (2004) observed that the microbial biomass of freshwater sediments was affected by the pesticides deltamethrin and isoproturon in concentrations in the range of $\mu\text{g per kg}$ of dry sediment, which are similar to the $15 \mu\text{g kg}^{-1}$ used in the present study. Divergent data have been published about the effects of organic pollutant on microbial biomass, because the observed effects may depend on the method used as well as on a variety of physical and chemical characteristics of the studied compound and environmental conditions. This is the first study, so far, which investigates effects of steroids on microbial biomass.

4.2. Effects of TBOH on microbial community structure

4.2.1. Microbial community structure assessed by 16S rDNA and RAPD fingerprints

Even though, similar cell densities were detected in control and treated microcosm, microbial community structure might have been altered due to TBOH treatment, as some microorganisms may be suppressed and others may proliferate in the vacant ecological niches without changing the total cell number. Therefore, effects of TBOH on bacterial and archaeal community structure as well as the total genetic diversity were also investigated.

DGGE analyses of 16S rDNA fragments indicate that the structure of the bacterial community was not altered by TBOH treatment. Using the same approach, Jontofsohn et al (2002a) did not detect effects of nonylphenol on bacterial communities. However, β -Proteobacteria and γ -Proteobacteria numbers increased in nonylphenol-treated microcosm, as detected by

fluorescence in situ hybridization (Jontofsohn et al., 2002b). In the present study, it is also possible that although the same bacterial populations were present in control and treated microcosms, their relative numbers might have changed due to trenbolone treatment, without being detected by the used method.

Different studies have shown distinct sensitivity of DGGE analysis for the detection of changes on bacterial community structure caused by pollutants. The application of 1 mg L⁻¹ alkylbenzene sulfonates in aquatic microcosms clearly affected the bacterial community structure, as showed by the DGGE band profiles (Brandt et al., 2004). In the same study, changes on substrate utilization potential only occurred when the microcosms where treated with 100 mg L⁻¹. Similarly, distinct band profiles were obtained for soils with different degree of mercury contamination (Müller et al., 2001b). In both studies, DGGE analysis was described as one of the most sensitive parameter for the detection of effects of pollutant on bacterial communities. In contrast, a study conducted by Ellis et al (2003) showed that in soils with different patterns of heavy metal contamination, even though the cultivable fraction of the community was very distinct, DGGE profiles remained very similar. In this and other studies, the analysis of total bacterial communities by DGGE was criticized (Griffiths et al., 2003; Siciliano et al., 2003) because of its lack of sensitivity.

As archaeal and bacterial populations may differ in their reaction to environmental stress, effects of TBOH on archaeal community was investigated by DGGE. In fact, in reactors containing pharmaceutical wastewater, changes on the bacterial community structure were observed while overall archaeal diversity remained very similar under various influent compositions (Oz et al., 2003). In the present study, no differences were detected between control and TBOH treated samples, corroborating the results obtained for the bacterial community. As archaea was previously associated to extreme environments, less comparative data is available in the literature about effects of organic pollutants on these organisms. In a landfill leachate-polluted aquifer, DGGE analyses of 16S fragments revealed clear differences between the structure of archaeal and bacterial communities inside and outside the contaminant plume (Röling et al., 2001). In contrast to the groundwater, no relationship to pollution was apparent from the analysis of the microbial community structure of sediment. The authors suggested that microbial community present in the sediment may be physically (e.g., in pores) or biologically (e.g., in biofilms) protected from the influence of leachate. Moreover, the great heterogeneity of the sediment could have impaired the detection of

changes on microbial communities caused by the leachate (Röling et al., 2001). High heterogeneity cannot explain the lack of effects of TBOH on microbial communities, as the sediment was well mixed before used in the microcosm experiment. However, other in situ factors, such as the binding of TBOH to sediment organic matter, may reduce its toxic effects to microbes (Eggleton and Thomas, 2004).

In theory, species diversity should be a more sensitive indicator of the effects of environmental pollutants than functional parameters, such as process rates, since the replacement of sensitive species by more tolerant species does not necessarily cause changes on microbial community function (Johnsen et al., 2001). However, a detailed investigation of microbial species diversity is very laborious and no available method can cover the whole microbial diversity present in an environmental sample. Therefore, some changes in the microbial community structure may remain undetected. DGGE analyses are considered to cover the dominant fraction of the microbial community. In this case effects on non-dominant organisms are probably overlooked.

As no TBOH-induced changes were detected by 16S rRNA gene analyses, microbial communities from laboratory experiment were also investigated by RAPD. This method employs primer of arbitrary sequences which allow the amplification of matching sequences within a DNA sample by PCR, resulting in a genetic fingerprint of the community. RAPD analysis gives a more broad view of the community as it does not focus in certain organisms or genes (Franklin et al., 1999). As indicated by the RAPD fingerprints, DNA sequence diversity of sediment microbial communities was not affected by TBOH treatment, corroborating the results obtained by the DGGE analyses. Similarly, the application of 2,4-D did not altered total genetic diversity from microbial communities of soils, with or without history of exposure to this herbicide (Xia et al., 1995). Nevertheless, comparisons between a soil contaminated with pesticides (triazolone and intermediates) and an untreated soil, which possess the same physical-chemical characteristics, showed that although the RAPD fragment richness was the same in both samples, their total genetic diversity clearly differed (Yang et al., 2000). The long-term contamination of the soil with high concentration of pollutants may have caused changes in the microbial community. In contrast, even though the concentration used in the present study might have been high compared to the concentrations normally found in the environment, it was probably too low to cause changes in the microbial

community structure. Moreover, the exposure time might have been too short to allow the detection of alterations of the microbial community structure.

4.2.2. Bacterial diversity assessed by clone libraries

To rule out possible limitations of DGGE and to obtain qualitative information about the microbial community present in the sediment, 16S rDNA clone libraries were generated for control and TBOH treated samples. Although the rarefaction analyses were made at phylum level and not operational taxonomic units, bacterial diversity was not fully covered in both clone libraries. This result was expected, since sediments are known to possess highly heterogeneous environmental conditions even in small scale and are, therefore, assumed to hold high microbial diversity (Bosshard et al., 2000; Madrid et al., 2001; Miskin et al., 1999). Similarly to other aquatic environments, 16S rDNA clone libraries of both control and trenbolone treated samples were dominated by sequences affiliated to *Proteobacteria* (Bosshard et al., 2000; Hiorns et al., 1997). The β -class, which is frequently detected in freshwater sediments and their overlying water column (Glökner et al., 1999; Spring et al., 2000), represented the majority of the *Proteobacteria* detected in the libraries. As in the present study, Hiorns et al (1997) observed that, after β -*Proteobacteria*, sequences related to γ - and α - *Proteobacteria* dominated mountain lake clone libraries by representing, respectively, 19 and 9 % of the detected clones. Although, α - and γ -*Proteobacteria* were found in both control and TBOH treated libraries, the representativeness of these groups differ among the libraries. Members of the δ -*Proteobacteria* which are more often found in sediments than in water columns (Spring et al., 2000), were not detected in this study. Normally analyses of sediments are done at the deepest part of the lake, where environmental conditions in the sediment greatly differ from the water column. As sediment samples were collected from a littoral area of the Lake Ammersee, the water column and sediment surface might be more similar to each other than observed in other investigations.

The main feature of the bacteria communities analyzed was the abundance of sequences types affiliated to the β -*Proteobacteria* and related to the genus *Burkholderia*. Many strains belonging to this genus, such as *B. cepacia* LB400, are shown to degrade a broad range of organic pollutants (Arnett et al., 2000; Kilbane et al., 1982). *Burkholderia*-related sequences dominated clone libraries from a soil contaminated with polychlorinated biphenyl (Nogales et al., 2001). In a study performed with a laboratory-scale bioreactor, bacteria detected using

probes targeting *B. cepacia* and *B. vietnamiensis* sequences were the main degraders of a mixture of polyalkylated aromatic compounds (Stoffels et al., 1998). Jontonfsohn (2002) also found *Burkholderia*-like sequences in clone libraries from microcosms filled with Lake Ammersee sediment. These sequences were affiliated to the strain CS-K2 isolated from Lake Chiemsee sediment. The strain CS-K2, most closely associated to *B. cepacia*, represented more than 20 % of sequences obtained from the littoral area of the lake (Spring et al., 2000).

Moreover, other members of the *Comamonaceae* family are known to degrade steroids. *Comamonas testosteroni* grows rapidly on steroids, such as testosterone, as its sole carbon source and under suitable conditions degrades these compounds to CO₂ and water by enzymes which are known to be steroid-induced (Maser et al., 2001; Xiong et al., 2001). Although, sequences related to this organism were not found in the libraries, it is possible that other uncultured members of the β -*Proteobacteria* also possess the ability to degrade steroids.

Many clones detected in both libraries were related to sequences of ammonia-oxidizing bacteria, mainly to *Nitrosococcus* sp. a member of γ -*Proteobacteria* class. These bacteria, which oxidize ammonia to nitrite as an energy source and assimilate CO₂ as the major carbon source, are capable of cooxidizing a broad range of hydrocarbons, such as naphthalene (Chang et al., 2002), chloroethane (Rasche et al., 1990), methylbromide (Duddleston et al., 2000). Moreover, in nitrifying activated sludge reactors, the steroid hormone ethinylestradiol was oxidized at maximum rate of 1 $\mu\text{g g}^{-1} \text{h}^{-1}$ resulting in the formation of hydrophilic compounds (Vader et al., 2000).

Knowledge of the diversity of microbial communities inhabiting polluted environments is useful since it provides clues about the type of bacteria able to adapt to and to exploit such habitats. In the present study the analyses of the clone libraries suggest that bacterial communities found in the microcosm have the potential to degrade substances which are structurally similar TBOH.

4.3. Effects of TBOH on active bacterial populations

The use of 16S rRNA genes for the investigation of effects of environmental stress, such as caused by pollutant, on bacterial communities has been criticized, as in DNA-based

approaches bacterial populations are detected irrespective to their metabolic state. Moreover, DNA of dead cells may be very stable in the environment (Demaneche et al., 2001) and, therefore, be included in the analyses. 16S rRNA analyses are considered to better represent the metabolic active fraction of bacterial community, as several studies have demonstrated a strong positive relationship between ribosomal content of bacterial cells and their metabolic activity under laboratory conditions (Kemp, 1995). Besides, RNA molecules are more prone to nuclease degradation than DNA and therefore are less stable in the environment (Duarte et al., 1998).

16S rRNA fingerprints, which were more than 90% similar to 16S rDNA fingerprints, also did not show changes on bacterial communities due to TBOH treatment. Dominant populations of the total community, detected by the 16S rDNA fingerprints, could be interpreted as metabolic active. However, low total activity and high bacterial numbers has led to the assumption that the proportion of active cells in sediments is usually low (Novitsky, 1987). In the present study, the low overall microbial activity, in spite of the high cell numbers, confirms this hypothesis. Increases in the rRNA contents of active cells may be masked by the more abundant rRNA from dormant cells. Small bacterial cells do not greatly contribute to metabolic activity in aquatic environments (Bernard et al., 2000), while they may carry a large proportion of the genetic material.

The use of BrdU for the labeling and detection of DNA synthesizing cells should overcome the problems discussed above. No labeled DNA was recovery in samples without any nutrient supply, which indicates the absence or low number of growing cells and reinforces the assumption that the metabolic activity in the sediment was low. Similarly, Borneman (1999) only detected BrdU-labeled DNA in soil samples supplied with nutrients. In this study, although the whole community (16S rDNA) remained unaltered, fingerprints from BrdU-labeled DNA clearly distinguished among nutrient treatments.

No BrdU-labeled DNA was detected in sediment samples after incubation with 0.5 mg g⁻¹ TBOH without yeast extract supply. This result demonstrates that TBOH did not stimulate bacterial growth under the studied conditions. In an aquifer soil treated with ¹³C-naphthalene, labeled DNA from bacteria associated with naphthalene degradation could be detected after short incubation (Jeon et al., 2003). However, in contrast to the present study, those microorganisms were adapted to naphthalene as the soil has a long history of contamination.

The presence of others EDCs on the lake can not be discarded, as six sewage treatment plants are present in the catchment area of the Ammer, which brings great part of the water from the Ammersee. However, TBOH is not expected to be found in the Ammersee at environmental relevant concentrations (ng L^{-1}), as the use of this product in animal farming is not permitted in Germany. Nevertheless, although steroid substances are found in nature, synthetic compounds may be more recalcitrant to microbial degradation than their natural analogues (Ternes et al., 1999). Nevertheless, after 56 days of incubation, up to 21 % of the TBOH added to soils was mineralized by microorganisms (FDA, 1987). Therefore, in the present study, the incubation period was probably too short to allow the detection of TBOH degradation. Further experiments should be undertaken, using a system which holds up longer incubation, in order to clarify these inquiries.

The same band profiles were detected for sediment samples enriched with yeast extract and treated with no or 0.5 mg g^{-1} TBOH, indicating that the population composition did not change due to the treatment. This find was unexpected, as at lower concentrations ($\pm 16 \mu\text{g mL}^{-1}$) TBOH was shown to totally inhibit *Salmonella* growth (Lutz et al., 1988). Species employed in toxicity tests may not exhibit the same response of indigenous bacterial populations in an environmental sample (Eismann and Montuelle, 1999). Interestingly, the intensity of some bands differed among treated and untreated samples. Possibly TBOH treatment caused the reduction of more sensitive bacterial populations and consequently resistant or tolerant organisms may be stimulated due to decrease of competition and simultaneous release of organic matter. The exposure of microbial communities to the EDC nonylphenol led to the reduction of *Actinobacteria* and to the increase of the *Proteobacteria* numbers (Jontofsohn et al., 2002b; Lozada et al., 2004).

The main critic on the BrdU-based analyses of active microbial populations had been the incapacity of certain organisms to incorporate BrdU. In the present study *B. cepacia*, *A. citreus* and *Cytophaga* incorporated BrdU in pure culture, under the used experimental conditions. This was an important finding as *B. cepacia*-related organisms represented a large fraction of the clone from both libraries. The fact that *P. fluorescens* did not incorporate BrdU indicates that experimental conditions should be improved as other members of this genus were shown to efficiently incorporate BrdU (Pernthaler et al., 2002). Nevertheless, as demonstrated for *B. subtilis*, BrdU may be toxic to some microorganisms (Binnie and Coote, 1986).

4.4. Effects of TBOH on microbial community function

Microbial activity is often limited by the lack of suitable nutrients at natural conditions. Therefore, microbial activity measurements usually require the amendment of nutrient sources (Raubuch and Beese, 1999). In the present study, no stimulation of overall activity was observed after enrichment of the sediment with its own DOC, showing that organic and/or inorganic substrates were limiting in the system. This might have been the cause of the low metabolic activity detected in the sediment. The addition of yeast extract strongly enhanced microbial activity. Similarly, in marine sediments nutrient amendment led to a clear increase of the active bacterial fraction (Luna et al., 2002).

The addition of $15 \mu\text{g kg}^{-1}$ TBOH did not affect overall microbial activity. Prado and Airoidi (2001) observed a dose-dependent inhibition of soil microbial activity by the herbicide diuron, at concentrations higher than 1.67 mg kg^{-1} . However, at lower concentrations (maximal permissible concentrations) herbicides also did not affect microbial activity (Widenfalk et al., 2004). In the present study, a reduction of the overall activity should be expected as the catabolic potential of the community was affected by TBOH. Nevertheless, as sum-parameters reflect the combined responses of different populations in the community, they are generally less sensitive than other parameters for the detection of effects of pollutants (van Beelen and Doelman, 1997).

Contamination with mercury and trinitrotoluene, which are known to have toxic effects on microbes, caused the reduction of the substrate utilization rates of soil microbial communities (Müller et al., 2001a; Siciliano et al., 2000). Similarly, the present study shows a reduction in the number of carbon sources metabolized by microbial communities from TBOH treated microcosms. The fact that TBOH directly added to BIOLOG[®] plates also caused the reduction of the substrate utilization potential of microbial communities from non-treated microcosms indicates that TBOH directly affects microbial community functions. Furthermore, although the results could not be statistically proved, the reduction of the substrate utilization potential due to the addition of TBOH to the microcosms and directly in the plates strongly indicates the effects of this compound to the community.

Microbial communities from control and TBOH treated microcosm exhibit similar substrate utilization potential, when TBOH was directly added to the plates, showing that no tolerance

toward TBOH was detectable after 19 days treatment. Some authors postulate that pollution may lead to development of tolerance within a stressed community. According to this concept, a community exposed to a toxicant at sufficiently high concentrations would change its structure and tolerance because sensitive organisms with lower competitive ability would be suppressed by more resistant organisms (Blanck et al., 1988). Therefore, this result supports DGGE analyses, which did not detect changes in the microbial community structure. In the present work, the incubation time might have been too short to allow the development of pollution-induced community tolerance (PICT). However, Salminen et al. (2001) also did not observe PICT in soils with a long history of zinc contamination. This shows that evolution to a tolerant state may be not a common reaction to environmental pollution.

The reduction of the catabolic potential of the community suggested that enzyme activities may also be affected by TBOH. Therefore the effects of TBOH on the activity of enzymes involved in nitrogen, phosphate and carbon cycling were investigated in the small microcosms experiment. The median values of leucine-aminopeptidase and glucosaminidase activities were very similar in control and treated microcosms, indicating that TBOH did not affect the activity of these enzymes. Phosphatase activity was higher ($p > 0.05$) in TBOH treated samples. However, amount of phosphate was slightly reduced in the sediment of treated microcosms, which could explain the difference in the enzyme activity. Free phosphate inhibits phosphatase activity and is probably more important than other parameters such as DOC in the regulation of this enzyme (Montuelle and Volat, 1998). The reasons for reduced phosphate content in the microcosms treated with TBOH are not clear. N-acetylglucosaminidase (chitinase) activity was significantly ($P < 0.01$) higher in control microcosms. No comparative data are available from other studies on EDCs. However, a strong reduction of chitinase activity, in a lake sediment contaminated with organic halogens, was correlated with a decrease in chitin content, which may be due to reduction of zooplankton species diversity and abundance. This was shown for example in water bodies receiving pulp mill effluents containing organic halogens (Wittmann et al., 2000). Nevertheless, in order to ascertain that the same process occurred in the TBOH contaminated microcosms further studies must be done including the investigation of the zooplankton.

Moreover, environmental changes, such as caused by pollutants, can alter enzyme activity patterns (Kuhbier et al., 2002). As described by Boon (1990), contamination with sewage effluent enhanced protease activity and inhibited phosphatase activity due to the high input of

protein and inorganic phosphorus. In the present study, although TBOH reduced chitinase activity, the enzyme activity pattern was not altered. The following pattern was observed for both control and TBOH treated microcosms: phosphatase > leucine-aminopeptidase > β -glucosidase > N-acety-glucosaminidase. Other sediments presented similar patterns (Kuhbier et al., 2002). However, higher protease activities than phosphatase activities have been observed in sediments receiving sewage effluent. The decrease of inorganic phosphorus concentration detected in the Lake Ammersee during the last 2 decades (Nixdorf et al., 2004) could explain the high phosphatase activity in the sediment.

The reduction of chitinase activity in TBOH treated sediments encouraged the investigation of possible effects of this compound on chitinase degrading bacterial populations. Therefore, group A chitinase genes and their expression were studied in the laboratory microcosm experiment. Chitinase A genes could be detected in both, control and TBOH treated microcosms. However, no expression could be detected by RT-PCR. The low metabolic activity of the studied sediment may difficult the detection of gene expression.

No differences were observed between DGGE profiles of control and TBOH treated sample, indicating that this compound did not affect bacterial populations carrying on chitinase A genes. This result indicates that the effects of TBOH on chitinase activity were not associated with changes on chitin degrading bacterial populations.

Moreover, the high percentage of *Proteobacteria* sequences found in the clone libraries and the importance of denitrification in aquatic ecosystems encourage the study of possible effects of TBOH on expression of nitrite reductase genes *nir S* and *nir K*, which encode, respectively, for cytochrome *cd1* and Cu-containing nitrite reductases. Only *nir S* could be clearly detected in the sediment samples from the laboratory experiment. Although some clones showed high similarity to 16S sequences of denitrifying bacteria carrying on *nir K* genes, such as *Ochrobactrum anthropi*, a clear band of correct size was not detected in the PCR. As known so far, *nir S* genes are more widely distributed than *nir K*, which are found only in 30% of the denitrifying bacteria (Coyne et al., 1989). Nevertheless, *nir K* genes might have been present in the gene pool in numbers which do not allowed their detection by PCR. In other studies, both *nirS* and *nir K* were found in the gene pool of sediments (Braker et al., 1998).

The expression of nitrite reductase genes was not detected for both control and TBOH treated microcosms. The low metabolic activity in the sediment might difficult the detection of the

expression of functional genes. Nevertheless, environmental condition in the microcosms probably did not favor denitrification process. At the static conditions of the microcosms oxygen could have become limited along the incubation, which, therefore, inhibited nitrification and caused NH_4^+ accumulation and NO_3^- depletion. Therefore, as both low oxygen concentration and a nitrogen oxide must be present to the induction of denitrification genes (Zumft, 1997), their expression could not be observed under the environmental conditions of the microcosms.

Although the use of functional parameters for the detection of effects of pollutants, such as pesticides, on microbial communities was criticized (Johnsen et al., 2001), in the present study microbial community function was more sensitive to TBOH treatment than the structure. In the literature similar comparisons have shown contrasting results. In soils contaminated with mercury, microbial community structure was significantly altered, although the functional potential of the community was only slightly affected (Müller et al., 2001a). The authors described the stability of the function as a form to compensate the reduced population size and diversity. On the other hand, phytoremediation systems increased the catabolic activity of rhizosphere soils due to changes on the functional composition of the community, which were not observed for the structure (Siciliano et al., 2003). Similarly, studying the effects of water stress on grassland bacterial populations, Griffiths et al. (2003) showed changes on the substrate utilization potential, but not on the DGGE profiles of 16S rRNA amplicons.

4.5. Conclusions and perspectives

Microbial activities are essential for turnover processes in all ecosystems and microorganisms form the basis of the food web. Therefore effects of pollutants on microbial populations may have consequences to the whole ecosystem. Although many studies have shown that EDCs, e.g. steroid hormones, can be degraded during secondary treatments of STPs, these compounds are often detected in rivers and lakes world wide. This reinforces the importance of the present study, which dealt with interactions of EDCs and microorganisms under realistic environmental conditions. Moreover, studies on benthic microbial community are of especial interest as these chemicals are shown to accumulate in the sediment.

Overall, microcosms systems used for this study were valuable tools, as replicates from treated and untreated microcosms showed a reproducible development of microbial

community structure and functions. Furthermore, microcosms resembled the environmental conditions observed in the natural ecosystem. Effects of TBOH on non-target organisms were supposed to be irrelevant at concentrations which EDCs are normally found in the environment. However, the present study shows that TBOH affected microbial communities, in a short-term experiment, at environmentally relevant concentrations. Although microbial diversity have been considered more sensitive than other parameters for the detection of effects of pollutants, neither bacterial and archaeal community structure nor the total genetic diversity were affected by TBOH. In contrast, chitinase activity was almost 50 % lower in TBOH treated samples. Also, the substrate utilization potential was reduced after TBOH treatment. Interestingly, this potential did not recovered 19 after the addition of the chemical. This data demonstrates that investigations based exclusively on the structural analyses can mask important effects of pollutants.

The use of artificial systems has the great advantage of allowing the investigation effects of contaminants on microbial communities in comparison to untreated communities under identical conditions (sediment, temperature, etc...). Nevertheless, although the outdoor microcosms experiment ran for 4 months, and was described here as long-term investigation, the response of microbial communities to the environmental changes caused by contaminants should be investigated for longer periods (years), in order to elucidate the relevance of those changes in relation to naturally occurring variation. Moreover, a better understanding of microbial processes involved on EDCs degradation is essential to the reduction of the environmental damage caused by these substances.

5. Summary

The aim of the present study was to investigate possible effects of trenbolone (TBOH), a synthetic hormone used in cattle production, on the microbial community in the sediment from the Lake Ammersee, Southern Germany. An outdoor microcosm system, constituted by steel cylinders (\varnothing 80 cm, height 60 cm) containing sediment and water collected from a littoral area of the lake, was used to assess semi-long-term (over a period of 4 months) effects of TBOH on microbial community structure. In this experiment realistic exposure conditions in which EDCs are constantly released in aquatic ecosystems were simulated by the use of membrane-based controlled LDPE devices. Furthermore, short-term (over a period of 19 days) effects, caused by a single application of TBOH, on microbial community structure and functions were determined in laboratory-scale microcosms (\varnothing 15 cm, height 25 cm). The structure of archaeal and bacterial communities as well as total genetic diversity, assessed by 16S rDNA and RAPD fingerprint analyses, respectively, were not affected by TBOH. Similarly to other aquatic ecosystems, 16S rDNA clone libraries generated from both treated and untreated microcosm were dominated by sequences related to β -Proteobacteria. The high number of clones affiliated to *Burkholderia* sequences detected in the libraries was a very interesting finding as members of this genus are known to degrade a variety of organic pollutants. Moreover, many clones were related to the ammonia-oxidizing bacteria. These bacteria are known to co-oxidize many organic compounds, including the steroid hormone ethinylestradiol. Analysis of 5-deoxybromouridine labeled DNA, which allowed the detection of DNA synthesizing cells, was used as an alternative method for the detection of metabolic active cells. Even though TBOH was applied in very high concentrations, changes of composition of active bacterial population were not observed. Nevertheless, although the overall microbial activity was not changed, the substrate utilization potential, measured using the BIOLOG system, was reduced due to TBOH treatment. Microbial communities from control and TBOH treated microcosm exhibit similar substrate utilization potential, when TBOH was directly added to the plates, showing that tolerance toward TBOH was not detectable after 19 days treatment. Moreover, N-acetylglucosamine (chitinase) activity was almost 50 % lower in TBOH treated samples. As shown by the DGGE profiles of chitinase genes (*chi A*), these effects could not be associated to changes on chitin-degrading bacterial populations. Overall the results indicate that microbial community function was more sensitive to TBOH treatment than the community structure.

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