EFFECTS OF ZINC PYRITHIONE AND COPPER PYRITHIONE ON MICROBIAL COMMUNITY FUNCTION AND STRUCTURE IN SEDIMENTS

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Abstract—The effects of the new antifouling biocides, zinc pyrithione (ZPT) and copper pyrithione (CPT), on microbial communities in estuarine sediments were studied in microcosms. As functional endpoints, fluxes of nutrients (NO\textsubscript{3}\textsuperscript{−}, NH\textsubscript{4}\textsuperscript{+}, HPO\textsubscript{4}\textsuperscript{2−}, Si(OH)\textsubscript{4}) and protein synthesis ([\textsuperscript{14}C]leucine incorporation) were used, whereas molecular fingerprinting methods (polymerase chain reaction/denaturing gradient gel electrophoresis) were used to describe the bacterial community structure. The lowest-observed-effect concentration (LOEC) for ZPT was 0.001 nmol/g dry sediment for the phosphate flux and total DNA content, whereas the LOEC for CPT was 0.1 nmol/g dry sediment for the nitrate flux and total DNA content. Nitrate fluxes increased significantly following additions of both ZPT and CPT, whereas ammonium fluxes decreased significantly after ZPT addition, suggesting changes in the nitrification and denitrification processes. The total DNA content decreased significantly following addition of both ZPT and CPT, but at the highest addition of ZPT (10 nmol ZPT/g dry sediment), an increase in total DNA content was found. Increased protein synthesis and bacterial diversity were also observed at this concentration of ZPT, suggesting growth of tolerant opportunistic species.

Keywords—Zinc/copper pyrithione Microbial community structure/function Nitrogen cycling

INTRODUCTION

The need for new antifouling paint biocides for leisure boats is increasing since the ban on tributyltin (TBT) as the active biocide in such paints was widely imposed in 1990s, especially by the Marine Environmental Protection Committee of the International Maritime Organization. Two new alternatives to TBT, zinc pyrithione (ZPT) and copper pyrithione (CPT), were introduced on the market (in 1991 and 1996, respectively) by Arch Chemicals (Norwalk, CT, USA), but only limited research has been conducted to assess the fate and effects of ZPT and CPT in the environment.

Arch Chemicals showed, through standard laboratory toxicity tests, that ZPT affected a wide range of organisms. Algae were affected at 18 μg/L (57 nM), crustacea at 11 μg/L (35 nM), adult fish at 1.6 μg/L (5 nM), early life stages of fish at 1.1 μg/L (3.5 nM), and oysters at 10 μg/L (31 nM); these values are lowest-observed-effect concentrations (LOECs) [1]. Furthermore, it was shown that pyrithione inhibited the growth of both a diatom (Phaeodactylum tricornutum) and a marine bacterium (Pseudomonas marina) at a concentration of 15 ppb [2]. Limited work has been done on CPT, but it has been shown that CPT is more toxic than ZPT to fish cells [3]. However, it was recently shown that ZPT (no-observed-effect concentration [NOEC], 0.01 fg/L) is more toxic than CPT (NOEC, 1 pg/L) to the development of sea urchins [4].

All the above-mentioned tests have been carried out as single-species tests. Such tests have been argued to provide only one perspective in risk assessment, because the environmental complexity is not taken into consideration [5]. In contrast, studies involving multiple species and whole communities have been argued to provide responses that are more comprehensive indicators of overall toxicity in the environment [6,7]. In ecotoxicological experiments on whole communities, diversity is a key parameter to analyze, because alterations in diversity have been hypothesized to have negative effects on the stability and function of a community in a given environment [8]. The overall function of a community consisting of generalists and specialists can, indeed, be changed if a perturbation affects, for example, the specialists, which are often the most vulnerable, because little or no redundancy is found in the specialized processes they perform. Often, generalists rely on the specialists to perform certain processes/reactions, which can be exemplified by the relationship between nitrifiers (specialists) and denitrifiers (generalists), in which denitrifiers rely on the nitrifiers to produce nitrate. The introduction of a pollutant into an environment can impose a selection pressure, which may lead to alterations in the genetic diversity because of differences in the sensitivity toward the pollutant of the species in the particular environment [9]. This has been shown, for example, regarding TBT and macro- and meiofaunal communities in marine sediments [10], diuron and marine diatom communities [11], refinery effluent water and microbial communities [12], and copper and higher plants (Silene vulgaris) [13]. Several studies [14–17] have shown that pollutants also can alter the diversity of microbial communities. In these experiments, the introduced compounds reduced the diversity of the bacteria in the test systems.

Bacteria are ubiquitous, sensitive to toxicants [18], and important actors in the fundamental biogeochemical cycling of organic matter [19], all of which make bacteria relevant as effect indicators in ecotoxicological studies regarding sediments. By measuring fluxes resulting from heterotrophic degradation of organic matter and from autotrophic bacterial processes, an integrated view of the entire microbial degradation can be found, because fluxes of nutrients are net results of the metabolism of the entire sediment community [20,21]. Specific
and well-defined processes, such as nitrification and methane oxidation, which are carried out by a limited portion of the bacterial community, often are more sensitive to changes in diversity than are general processes carried out by a wide range of microorganisms [21]. This can be caused, in part, by the lack of functional redundancy in these processes. On the other hand, effects on general processes (e.g., ammonification) can be totally or partly hidden, because organisms that are not affected take over the functions of those that are affected [22]. It has, however, been shown that general parameters also can be sensitive to changes in the environment (e.g., incorporation of \[^3H\]thymidine [replication rate] [18] and changes in bacterial biomass [23]).

So far, no studies have concentrated on the effects of ZPT and CPT on bacteria in either marine or estuarine sediments. When ZPT reaches the sediment, it can accumulate as stable manganese [24] or CPT complexes [25]. Degradation of ZPT proceeds fast in anaerobic sediment (0.5 h for 50% degradation) but is especially slow in aerobic sediments (2-22 h for 50% degradation) [26]. To our knowledge, no information concerning the degradation rate of CPT has been published.

The purpose of the present study was to determine the effects of ZPT and CPT on bacteria in estuarine sediments during short-term experiments. We used the diversity of the gene coding for the RNA polymerase beta subunit (\(rpoB\)) as a community structure variable [27] and nutrient fluxes (NO\(_3^\), NH\(_4^+\), HPO\(_4^{2-}\), Si(OH)_4) and incorporation of \[^{14}C\]leucine into bacterial protein [28] as community function variables. Furthermore, the flux measurements of NO\(_3^\) were supported by flux measurements of traceable \(^{15}\)NO\(_3^\).

**MATERIALS AND METHODS**

**Sediment sampling**

Sediment was collected in October and November 2001 in a shallow part (depth, 0.5 m) of Roskilde Fjord (Roskilde, Denmark; 55°42′N, 12°6′E) at a place with no leisure boat activity. This allowed the assumption that the content of ZPT and CPT was negligible. Only the uppermost 2 cm of sediment were collected, sieved with a 0.5-mm polyethylene sieve to remove larger in-fauna, and kept in the dark at the in situ temperature of 10°C until the additions of ZPT and CPT. The water content of the sediment was 23%, and the organic content was 0.6%, as determined by loss on ignition.

**Addition of ZPT and CPT**

Stock solutions of zinc pyrithione (bis[1-hydroxy-2(1H)-pyridine-thionato]zinc or Zinc Omadine\(^\circ\)) and copper pyrithione (bis[1-hydroxy-2(1H)-pyridine-thionato]copper or Copper Omadine\(^\circ\)) (Arch Chemicals) were prepared in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany). Working solutions were prepared by adding 100 \(\mu\)l of stock solution to 100 ml of sterile, filtered seawater of the same salinity (10 psu) as that at the sampling site. Stock solutions and working solutions were kept in dark bottles to avoid photolysis. A working solution of 50 \(\mu\)l was added per gram wet sediment during constant stirring, giving a final concentration range from 0.1 nmol/g dry sediment to 10 nmol/g dry sediment (nominal concentrations). After addition of the working solution, the sediment (10 ml) was transferred to cylinder-shaped deldrin plugs (depth, 4 mm; width, 24 cm; diameter, 5.5 cm), and the sediment surface was leveled. The increase of the water content because of the addition of working solution was 3%. For further information on the open plug–flux method, see Dahllof et al. [29]. Reference controls with DMSO and seawater were prepared in the same manner. The reference controls were used to analyze potential effects of the use of DMSO as a solvent. No effect was observed for either the functional or structural endpoints, and the reference controls will not be further discussed. All chemicals were supplied by Sigma Chemical (St. Louis, MO, USA) unless otherwise mentioned.

**Incubation**

Plugs with sediment containing the different concentrations of added ZPT or CPT were placed randomly in two large containers (82.5 L), separating the plugs containing ZPT or CPT. Subsequent treatments and analyses were also performed randomly to avoid experimental biases. The plugs were pre-incubated for 3 d, after which nutrient fluxes were measured over 4 h.

The water in the large container was stirred by continuous bubbling with air from four sources. This was done to ensure that aerobic processes would dominate in the sediment plugs, although anaerobic processes also would be present. The sediment was preincubated with ZPT and CPT in the dark for 3 d at 10°C. After the preincubation, the plugs with sediment were placed in individual polypropylene containers, and the overlying water was siphoned off leaving approximately 14 ml (height, 5 mm) of water on top of each plug open to the air. Water samples from the large container were taken and filtered through a 0.45-µm cellulose acetate filter for nutrient analysis to determine the starting concentrations of nutrients in the following incubation (\(C_t\)). The plugs were left for 4 h in the dark at 10°C. Afterward, the overlying water was removed with a syringe and filtered through a 0.45-µm cellulose acetate cell filter for nutrient analysis (\(C_t\)). The fluxes of nutrients (NO\(_3^\), NH\(_4^+\), HPO\(_4^{2-}\), Si(OH)_4) were calculated as the difference in nutrient concentration between \(C_t\) and \(C_{t0}\). The nutrient analyses were performed on a SANPLUS System Scalar autoanalyzer (Skalar, Breda, The Netherlands).

**\(^{15}\)NH\(_4^+\) addition experiment**

To investigate the oxidation of ammonium to nitrate and to determine whether denitrification was present in the sediment during incubation, a subsequent experiment with labeled ammonium was performed. The \(^{15}\)NH\(_4^+\)Cl was added to the container, giving a final concentration of 95 µM (99.1% Europa Scientific, Stable Isotope Research Products, Cheshire, UK). The \(^{15}\)NH\(_4^+\) was added to the container 24 h before the pre-incubation (3 d) ended, and after the 4 h of incubation while open to the air, samples (5 ml) were taken out and analyzed for the content of \(^{15}\)NO\(_3^\) and \(^{15}\)N\(_2\).

**Diversity analysis of the bacterial community**

After removing the overlying water at \(t_0\), 0.2 g of sediment from each plug was taken out for diversity analysis. The sampling was done with a polypropylene syringe with the tip cut off, making it possible to take a vertical sample through the sediment, thus taking a representative sample of the bacteria in the sediments regardless of a potential difference in depth distribution. Sediment samples were frozen in 1.5-ml, screwcap cryotubes until further processing.

**DNA extraction**

The DNA was extracted from the sediment by bead beating. The sediment was centrifuged, and the overlying water was
removed from the sediment before adding 1 ml of extraction buffer (400 μl of 6.25 M ammonium acetate, 100 μl of 1 M Tris [pH 8], 40 μl of 0.5 M ethylenediaminetetraacetic acid [pH 8], 80 μl of hexadecyl trimethyl ammonium bromide/NaCl, and 460 μl of Milli-Q® water [Millipore, Bedford, MA, USA]). Furthermore, 200 μl of silica beads (Biospec Products, Bartlesville, OK, USA), 0.015 g of acid-washed polyvinylpolyrolidilne, and 300 μl of chloroform:isoamylalcohol (24:1; Lab Scan [Dublin, Ireland] and Merck, respectively) were added to each centrifuge tube.

The samples were bead beaten at 5.5 m/s for 30 s in a BIOR-101 bead beater (Savant, Holbrook, NY, USA) followed by centrifugation of the supernatants for 20 min at 15,000 g. The supernatants were transferred to new Eppendorf tubes. Next, 3 M NaAC (1/10th of the volume) was added, and the tubes were topped up with isopropanol (minimum 0.6 x volume) followed by precipitation overnight at 4°C. The samples were centrifuged, the pellets cleaned with 75% ethanol, and the DNA reconstituted in Tris/ethylenediaminetetraacetic acid (TEDTA) buffer. The total DNA content in the samples was quantified with PicoGreen® (Molecular Probes, Leiden, The Netherlands) on a fluorescence spectrophotometer (Fluostar; BMG Labtechnologies, Offenburg, Germany) and diluted to a concentration of 50 ng/μl before polymerase chain reaction (PCR).

PCR-denaturing gradient gel electrophoresis conditions

A fragment of the gene coding for the RNA polymerase beta subunit (rpoB) was amplified for subsequent denaturing gradient gel electrophoresis (DGGE) analysis using the following primers: rpoB1698F (5’-AACATCGGTGTGATCAAC-3’) and rpoB2041R (5’-GGTGCTATGGTAACTCCAT-3’). A GC-clamp was added to the forward primer at the 5’-end (5’-CGCCCCCAGCCGCGCCGGCGGCGCG-CCGCGCCCGCCCGCC-3’). One advantage of using the gene coding for the fragment of the RNA polymerase beta subunit (rpoB) in community analysis is that the gene is only found in one copy in bacteria, in contrast to multiple copies of the 16S rDNA, which have been found in several species. Furthermore, the rpoB gene possesses the same key attributes as the 16S rDNA (e.g., it is common in all bacteria, contains conserved as well as variable regions, and therefore can be used as a phylogenic marker) [27].

Additionally, part of the gene that encodes the active-site polypeptide of ammonia monooxygenase (amoA) was amplifi ed to describe the diversity of nitrifiers in the samples. The primers used were amoA332F (5’-GGGTTTCTAGTGGTGGT-3’) and amoA4822R (5’-CCCTCTGKSAAACGCGCTCTTC) [30]. A GC-clamp was added to the forward primer at the 5’-end.

Reaction mixtures (20 μl) contained 2 μl of PCR buffer, 0.5 μl of dNTPs (20 pmol/μl of each), 0.5 μl of forward and reverse primer (25 pmol/μl), 0.5 μl of bovine serum albumin (10 mg/ml), 13 μl of Milli-Q water, 1 μl of JumpStartTM RedTaq™ (Sigma-Aldrich, St. Louis, MO, USA) polymerase, and 2 μl of sample DNA (50 ng/μl). The samples were incubated in a Hybaid PCR express thermal cycling machine (Franklin, MA, USA) using hot-start PCR with a program of 5 min at 94°C; amplification for 25 cycles, consisting of denaturing for 30 s at 94°C, annealing for 1 min at 50°C, and extension for 1.5 min at 72°C; and a final extension of 10 min at 72°C. Primers were supplied by DNA Technology A/S (Aarhus C, Denmark), dNTPs by Invitrogen (Invitrogen, Carlsbad, CA, USA), and PCR buffer and JumpStart RedTaq polymerase by Sigma-Aldrich (St. Louis, MO, USA).

The PCR products were run in a DGGE gel with a gradient of polyacrylamide (6–8%) parallel to a 35 to 55% linear denaturant gradient (100% denaturing gradient contains 7 M urea and 40 ml of formamide per 100 ml). Electrophoresis was run for 16 h at 70 V using the Bio-Rad D-code system (Hercules, CA, USA) in 0.5× Tris-acetate-EDTA buffer. The gels were stained with SYBR® Gold (Molecular Probes) in 1× tris-acetate-EDTA buffer (1:10,000) and analyzed in Kodak 1D Image Analysis Software (Rochester, NY, USA).

Bacterial protein synthesis (incubation with [14C]leucine)

Analysis of protein synthesis was performed following the removal of water, at C1, from the plugs. One sample (~0.2 g of sediment) from each plug was taken as described for diversity analysis, and 200 μl of 10 μM [14C]leucine (295 mCi mmol−1; Amersham, BioScience, Buckinghamshire, UK) were added to 1.5-ml, screw-cap cryotubes. This was followed by a short vortex to ensure an even distribution of the [14C]leucine in the sample. The samples were incubated at 10°C for 1 h. Three replicates were fixed with formalin before addition of [14C]leucine to determine the amount of abiotic binding to the sediment.

Protein extraction

We developed and verified a fast and easy method to extract proteins from bacteria in sediments for experiments with many replicates or samples (outlined below). This method is based on the rationale behind the method for DNA extraction, in which the cells were lysed by bead beating and proteins are extracted from the sediment and dissolved in an extraction buffer.

After incubation with [14C]leucine, the bacterial cells were killed by adding 20 μl of formalin (final concentration, 2%). The samples were vortexed and spun down, and the supernatants were discarded. The 0.7 ml of TEDTA buffer, 0.5 ml of 3% sodium lauryl sulfate, and 200 μl of silica beads were added, and the samples were bead beaten at 5.5 m/s for 30 s followed by a short spin (1 min). This procedure disrupted the bacterial cells, thereby releasing the proteins. The supernatants were transferred to 2-ml Eppendorf tubes, and 1 ml of 100% trichloroacetic acid–CCl3 COOH (Merck) and 20 μl of skimmed milk were added to enhance the precipitation of protein. Skim milk was prepared by adding 1 g of skimmed milk powder (Scharlau, Barcelona, Spain) to 20 ml of autoclaved Milli-Q water. The samples were centrifuged at 14,000 g at 20°C for 20 min to precipitate the proteins. Finally, the supernatants were removed, and 1 ml of Ecoscint A (National Diagnostics, Atlanta, GA, USA) was added to the pellets. Washing the pellet in TEDTA buffer did not further remove any unincorporated leucine (data not shown). After 24 h of storage at room temperature, the radioactive decay was measured directly in the Eppendorf tubes on a Beckman LS 1801 scintillation counter (Irvine, CA, USA).

The [14C]leucine incorporation method for bacteria in the sediment developed in the present study yielded a maximum incorporation rate of 15.7% ± 0.9% (mean ± 95% confidence interval) of the added leucine in a 1-h incubation at 20°C. Of this, 97% was incorporated into the precipitated protein, and 3% was abiotically absorbed to the sediment. The samples with CPT or ZPT additions and the matching controls were incubated for 1 h at 10°C, which yielded an incorporation of
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Fluxes of inorganic nitrogen compounds. The fluxes of nitrate from the sediment to the water increased with increased additions of ZPT and CPT. The LOEC for the nitrate flux was found in sediment with 1 nmol ZPT/g dry sediment, and this addition caused a threefold increase in NO$_3^-$ flux from the sediment to the water compared to the controls (Fig. 1a). The LOEC for CPT was found in sediments treated with 0.1 nmol CPT/g dry sediment, which caused a fivefold increase in the flux of NO$_3^-$ from the sediment to the water compared to the controls (Fig. 1b).

The fluxes of ammonium were significantly affected by the addition of ZPT, but not by the addition of CPT (Fig. 1a). A decrease in ammonium flux was seen at concentrations of 1 and 10 nmol ZPT/g dry sediment (Fig. 1a). The measured flux rates are shown in Table 1.

Table 1. Flux measurements (μmol/m$^2$/d) as a function of zinc pyrithione (ZPT) and copper pyrithione (CPT) additions

<table>
<thead>
<tr>
<th>ZPT</th>
<th>Control sediment</th>
<th>1 nmol ZPT/g dry sediment</th>
<th>Δ Flux (control, 1 nmol)</th>
<th>10 nmol ZPT/g dry sediment</th>
<th>Δ Flux (control, 10 nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_3^-$</td>
<td>27 ± 13</td>
<td>83 ± 9*</td>
<td>56 ± 21</td>
<td>115 ± 5*</td>
<td>89 ± 18</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>397 ± 8</td>
<td>360 ± 6*</td>
<td>36 ± 14</td>
<td>345 ± 5*</td>
<td>52 ± 22</td>
</tr>
<tr>
<td>CPT</td>
<td>Control sediment</td>
<td>0.1 nmol CPT/g dry sediment</td>
<td>Δ Flux (control, 0.1 nmol)</td>
<td>1 nmol CPT/g dry sediment</td>
<td>Δ Flux (control, 1 nmol)</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>9 ± 5</td>
<td>49 ± 5*</td>
<td>40 ± 10</td>
<td>73 ± 13*</td>
<td>64 ± 18</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>319 ± 29</td>
<td>289 ± 37</td>
<td>30 ± 66</td>
<td>327 ± 26</td>
<td>8 ± 55</td>
</tr>
</tbody>
</table>

*The values are given as averages ± 95% confidence intervals. The concentration of NO$_3^-$ was 10 μM and the concentration of NH$_4^+$ 5.7 μM in the overlying water for ZPT and CPT incubations. Numbers marked with an asterisk describe values that are different from the controls on the 95% level (*p < 0.05).
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Fig. 2. Effect on the phosphate flux from the sediment as a function of zinc pyrithione (ZPT) addition. Mean values with 95% confidence intervals (n = 4) are shown. The left y-axis describes the fluxes of phosphate as a percentage of control, whereas the right y-axis shows the fluxes as μmol/m²/d. The concentration of phosphate was 0.68 μM in the overlying water for ZPT and copper pyrithione incubations. *p < 0.05.

Bacterial protein synthesis

The addition of ZPT affected the protein synthesis of the bacteria significantly at a concentration of 10 nmol/g dry sediment. At this concentration, the increase in the incorporation of [¹⁴C]leucine was 131% ± 5% of the controls (data not shown).

Effects of ZPT and CPT on structural variables

Total DNA content. A significant decrease in the total DNA content was seen at the lowest concentration of ZPT (0.001 nmol/g dry sediment). At the addition of 10 nmol ZPT/g dry sediment, a DNA content similar to that of the control was found. Similarly, the DNA content decreased significantly following addition of 0.1 and 1 nmol CPT/g dry sediment, but no increase in total DNA content at higher additions was observed (Fig. 3).

Bacterial diversity. The addition of 10 nmol ZPT/g dry sediment resulted in at least one extra band on the DGGE gel (Fig. 4). No differences were seen on the DGGE gel between the control sediment and the sediment with additions of CPT (data not shown). Furthermore, no differences in diversity were seen in the different treatments with ZPT or CPT using the gene coding for ammonia monoxygenase (amoA; data not shown).

A second microcosm experiment was performed to verify the obtained results for the functional variables, and similar results were found. The nitrate flux increased to 172% ± 22% of the control, and the ammonium flux decreased to 74% ± 7% of the control after the addition of 1 nmol ZPT/g dry sediment. Following addition of 0.1 nmol CPT/g dry sediment, the nitrate flux increased to 174% ± 17% of the control, but again, no significant effect on the ammonium flux was detected. The increase in protein synthesis was seen at 1 nmol ZPT/g dry sediment, and no significant effects were seen on the bacterial protein synthesis when CPT was added to the sediment.

DISCUSSION

Nutrient fluxes

The LOECs for the nitrate fluxes were observed at 1 nmol ZPT/g dry sediment and 0.1 nmol CPT/g dry sediment. The increase in the nitrate flux could be explained by an increase in nitrification (NH₄⁺ → NO₂⁻ → NO₃⁻), a decrease in denitrification (NO₃⁻ → N₂), or a combination of both. Because ammonium is the substrate for nitrification, a decrease in the ammonium flux would be expected when nitrification increases, which is what was observed in the experiments with ZPT. This makes it possible to calculate the effect of ZPT on both nitrification and denitrification, under the assumption that the general processes of ammonium assimilation and ammonification are unaffected.

The drop in ammonium flux between the controls and the addition of 1 nmol ZPT/g dry sediment was 36.6 μmol/m²/d,
and the increase in the nitrate flux was 56.5 μmol/m²/d (Table 1). This indicates that effects on nitrification can explain 65% of the increase in the nitrate flux; the remaining 35% can then be referred to as changes in denitrification processes. The same pattern was seen at the addition of 10 nmol ZPT/g dry sediment, in which an increase in nitrification of 60% could explain the increased nitrate flux and decreased ammonium flux. A decrease in the denitrification processes (40%) could explain the remaining part of the increase in nitrate flux.

Denitrification processes were mostly affected at the highest concentrations (10 nmol ZPT/g dry sediment and 1 nmol CPT/g dry sediment) of the toxicants, indicating that nitrification is a more sensitive parameter than denitrification in relation to the toxicity of ZPT and CPT. In particular, gram-negative cells are susceptible to the high membrane activity of pyrithione ions [33], which could explain the pronounced effect of ZPT and CPT on nitrifying bacteria, which are characterized as being gram-negative [34]. Denitrifying bacteria, on the other hand, are characterized as being both gram-negative and gram-positive [35]. Another explanation for the high sensitivity of nitrification compared to that of denitrification could be that denitrification is a general process that is performed by a wide range of bacterial species, whereas nitrification is performed by a few specialized species. This makes denitrification a process with high redundancy, so effects on denitrifying bacteria can be difficult to track.

Differences in substrate (NH₄⁺) availability could also explain the increased nitrification following ZPT and CPT additions. The amount of dissolved organic nitrogen (DON) is likely to be higher at high biocide concentrations because of leakage from dead cells. The increased amount of DON could enhance ammonification (DON → NH₄⁺), thereby increasing substrate availability, which in the calculations above was assumed to be unaffected. This would yield a higher amount of nitrate and, eventually, increase the nitrate flux. To test the hypothesis of increased substrate availability, [15N]ammonium was added to both the control and the treatment using 1 nmol ZPT/g dry sediment, giving a final concentration of 95 μM [15N]ammonium. At this concentration, the sediment was regarded as being substrate saturated. The increase in nitrate flux at 1 nmol ZPT/g dry sediment was significantly higher compared to the control in this experiment; thus, an increased nitrate flux could only result from a stimulation of the nitrification or a decreased denitrification and could not have been caused by an increased substrate availability. Ammonification generally appears to be unaffected, or even stimulated, by toxic compounds [36], which could be caused by a high degree of functional redundancy.

The experiments with CPT also showed a significant increase in the nitrate flux, but no concurrent significant drop in ammonium flux was found (Table 1). It should be noted in the CPT experiments that the variation in the ammonium fluxes was very high compared to that in the ammonium fluxes in the ZPT experiment. This high variability could have hidden, eventual effects on the ammonium fluxes in the CPT experiment. Because of uncertainties in the ammonium measurements, it is not possible to conclude whether the increase in nitrate flux is caused by increased nitrification or decreased denitrification. It could, however, be hypothesized that the mode of actions of ZPT and CPT are similar, implying that the increased nitrate flux could be explained by a combination of increased nitrification and decreased denitrification.

The increases in nitrification following additions of ZPT and CPT are very consistent with the mode of action of pyrithiones on membranes. Pyrithiones exhibit external, but mostly internal, membrane effects, which inhibit the primary proton pump [37]. Furthermore, pyrithiones disrupt membrane structure, inhibit the membrane-bound metabolic processes, and decrease the intracellular adenosine triphosphate (ATP) levels [38]. In comparison, it has been observed that the breakdown of glucose (i.e., catabolism) increases with increasing pyrithione concentrations. This increase was explained by the loss of intracellular metabolic control mechanisms caused by loss of membrane-related pH gradients, concentration gradients, transportases, and ATPases [39].

For the nitrifying bacteria to compensate for decreased intracellular ATP levels caused by the actions of the pyrithiones, a decoupling and an increase of the nitrification occur, but little or no ATP is produced. An increase in nitrification was also found following TBT addition to sediment, resulting in an increased nitrate flux and a decreased ammonium flux [29]. It could be expected that the nitrate flux would decrease over time, because it would be detrimental for the bacteria if little or no ATP were gained during nitrification. This pattern has been observed when sediments were exposed to TBT in long-term experiments [20].

The most sensitive endpoint measured in this experiment was the phosphate flux, which increased significantly at 0.001 nmol ZPT/g dry sediment. An increase in phosphate flux can result from an increased release of abiotically bound phosphate from the sediment because of the occurrence of anaerobic microzones and degradation of phosphor-containing organic matter. Such anaerobic microzones likely were created by the death and degradation of algal cells, which is supported by the reduction of total DNA in the same samples.

**Protein synthesis**

It could be expected that additions of ZPT and CPT would inhibit protein synthesis and, thereby, indirectly inhibit the growth of bacteria because of the membrane action of the toxicants. It has been found that pyrithiones inhibited the growth of a wide range of bacteria, especially gram-positive bacteria and, to a lesser extent, gram-negative bacteria [40]. However, no inhibition of protein production was found in the present study. On the contrary, an increase in protein production was seen at the highest concentration of ZPT (10 nmol/g dry sediment; 145% ± 10% of the control). This indicates that certain bacteria are capable of survival and incorporation of [14C]leucine at a high rate despite the addition of ZPT. At this ZPT load, favorable growth conditions, such as increased nutrient levels and decreased competition, would be present for tolerant bacteria, and these factors could, to a certain degree, explain the high protein production found at the highest ZPT concentration. It has further been reported that resistance to pyrithiones can occur in bacterial cells [40].

The incorporation of [14C]leucine was an insensitive endpoint to low additions of ZPT and CPT compared to flux measurements. Incorporation of [14C]leucine is a general process that is performed by a wide range of microorganisms; thus, it can be assumed that the degree of functional redundancy in this process is very high.

**Bacterial diversity and total DNA content**

Diversity analysis of the bacterial DNA in the sediment showed that one extra band appeared on the DGGE gel at the addition of 10 nmol ZPT/g dry sediment (Fig. 4). This finding
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could be correlated to the increased incorporation of $[^{14}C]l$eucine at this concentration of ZPT. Furthermore, the content of total extracted DNA initially decreased, followed by an increase at the addition of 10 nmol ZPT/g dry sediment, compared to the other concentrations of ZPT. It should, however, be mentioned that the total extracted DNA includes DNA from algae, bacteria, and fauna in the sediment, so an increase in total DNA cannot directly be interpreted as an increase in the amount of bacterial DNA.

The altered conditions in the sediment after the addition of 10 nmol ZPT/g dry sediment can give the opportunity for opportunistic bacteria to grow. The replacement of sensitive with tolerant species can have ecological consequences, because sensitive species could perform specific functions in the community that would be difficult to maintain if they disappear [41]. This means that an increase as well as a decrease in species richness should be considered as a perturbation.

Bacterial diversity would be expected to decrease after the addition of toxicants, because the most sensitive species would be eliminated in a heterogeneous population [42]. Such an effect, however, was not seen in the present study, probably because of the persistence of extracellular DNA from dead cells in the sediment. It has been reported that DNA was persistent in soil for weeks after its release from dead cells [43]. Persistent extracellular DNA from dead cells can hide the short-term effects of a toxicant on the diversity of bacteria, because it is amplified during PCR together with the DNA extracted from the surviving bacteria [44]. To distinguish active cells from dead cells in sediments, mRNA could be extracted and used to analyze the active part of the bacterial community in diversity analysis. Because mRNA from dead cells is single-stranded and more quickly broken down compared to double-stranded DNA, it would not be represented in the diversity analysis.

The increased nitrate flux from the sediment and the increased protein synthesis at the highest addition of ZPT could, theoretically, be explained by growth of nitrifying bacteria, which at the same time would explain the extra band appearing on the DGGE gel. To test this, the gene coding for ammonia monooxygenase (amoA) from the samples was amplified and run on a DGGE gel. This showed no differences between the treatments with ZPT, CPT, and the control, suggesting that the increase in growth and nitrate fluxes were not caused by an increased abundance of nitrifiers. However, taking into account the persistence of extracellular DNA and that the amoA primers do not catch all known nitrifiers (e.g., *Nitrosococcus oceanus* [30]), growth of nitrifiers cannot be totally excluded. The initial decrease in the total DNA content can be the result of bacterial breakdown of dead algal cells, whereas this degradation would not be so pronounced at higher concentrations of the biocides.

**CONCLUSION**

Clearly, the stress imposed by ZPT and CPT affected the function and structure of the microbial sediment community. The same effects can be expected in natural systems, because the set-up in these experiments, to a higher degree than in single-species tests, simulates the complexity of natural conditions. One of the most pronounced effects of ZPT and CPT was found on the nitrogen cycle, which is very important both for recirculating nitrogen to primary producers and for decreasing inorganic nitrogen via denitrification processes. It is possible that similar effects will be observed in nature when ZPT and CPT accumulate in the sediment. The changes observed at the molecular level (i.e., changes in the amount of extracted DNA and changes in diversity) could have adverse effects on the sediment community and, thereby, on the function of coastal sediments.

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