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C. Giray
University of Maine

G. M. King
University of Maine

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Effect of naturally occurring bromophenols on sulfate reduction and ammonia oxidation in intertidal sediments

C. Giray, G. M. King*

Darling Marine Center, University of Maine, Walpole, Maine 04573, USA

ABSTRACT: We examined the effect on ammonium oxidation and sulfate reduction of several brominated compounds [4-bromophenol, 2,4-dibromophenol (2,4-DBP), 2,6-dibromophenol and 2,4,6-tribromophenol] that occur naturally in enteropneusts. We compared rates of these processes with and without bromoorganics using bulk intertidal sediments and burrow wall sediments from 3 enteropneust species (2 containing 2,4-DBP and 1 containing 2,3,4-tribromopyrrole), a mollusc (*Mya arenaria*) and a polychaete (*Nereis virens*). Sulfate reduction in bulk sediment was unaffected by bromophenols at concentrations $<100 \text{ nmol cm}^{-3}$ sediment, but was inhibited temporarily at $1 \mu\text{mol cm}^{-3}$. Sulfate reduction in burrow wall sediments differed from bulk sediments, but was not correlated with concentrations of naturally occurring bromophenols. Ammonium oxidation was inhibited in surface sediments by bromophenol concentrations as low as 1 nmol cm^{-3} sediment. Ammonium oxidation was enhanced in burrow wall sediments of *N. virens*, *M. arenaria* and the enteropneust *Saccoglossus bromophenolosus* relative to surface sediments, but was markedly lower and negatively correlated with ambient 2,4-DBP in burrow wall sediments of a second enteropneust, *Protoglossus graveolens*.

KEY WORDS: Marine biogeochemistry · Enteropneusts · Bromophenols · Sulfate reduction · Ammonium oxidation · Microbial inhibition

INTRODUCTION

Numerous volatile halogenated organic compounds have been described from many marine organisms, including sponges, polychaetes, phoronids, enteropneusts, algae and bacteria (see Neidleman & Geigert 1986, Corgiat et al. 1993, King et al. 1995 for references). A variety of functions have been proposed for these compounds, including predation deterrence (Prezant et al. 1981, Woodin et al. 1987, Teeyapant & Proksch 1993), microbial control (e.g. Ashworth & Cormier 1967, Sheikh & Djerassi 1975, Higa & Sakemi 1983, King 1986, 1988) and burrow conditioning (Nørrevang 1965, King 1986).

Although some evidence supports a role for haloorganics in microbial control, the effects of bromophenols on microbes vary depending on the group,

process or parameter examined. For example, 2,4-dibromophenol (2,4-DBP) inhibits glucose respiration by and growth of aerobic bacteria in sediment slurries and liquid culture at low concentrations (e.g. 10 nmol cm^{-3} ; King 1986, 1988). In contrast, 2,4-DBP has enhanced sulfate reduction rates, with only transient inhibition at mM concentrations (King 1988). Slightly higher bacterial densities occur in the burrow wall lining of *Stereobalanus canadensis*, a 2,4,6-tribromophenol (TBP)-containing enteropneust, than in adjacent sediments, but bacterial cell density, acetate assimilation, microalgal biomass and production, and meiofaunal densities do not differ significantly between bulk sediments and sediments from areas with *Notomastus lobatus*, a 4-bromophenol (BP)-containing capitellid polychaete (Steward et al. 1992). Similarly, no significant differences in microbial biomass and community structure have been noted for burrow sediments of *N. lobatus* and *Branchyosychus americana*, a polychaete that does not contain bromophenols (Steward et al. 1996).

* Addressee for correspondence.
E-mail: gking@maine.maine.edu

At least some of the differences among published reports may be attributed to differences in both the intrinsic toxicity of the various naturally occurring bromophenols and the susceptibility of specific taxa. In addition, biomass and community structure measurements may not be sensitive enough to detect small and general or substantial but specific impacts of bromophenols. Furthermore, comparisons of bromophenol impacts based on different animal taxa are likely to be confounded by differences in bromophenol content and excretion, as well as differences in burrowing habits, ventilation rates and types of sediment occupied.

We address here the relative effects of BP, 2,4-DBP, 2,6-dibromophenol (2,6-DBP) and TBP on 2 processes in bulk and burrow wall sediments. Each of these compounds has been reported for enteropneusts and some other taxa (e.g. King 1986, Woodin et al. 1987). 2,4-DBP appears particularly common in enteropneusts, occurring in both *Saccoglossus bromophenolosus* (King et al. 1994) and *Protoglossus graveolens* (Giray & King 1996). Enteropneusts, like other macrofauna, have a variety of biogeochemical impacts in sediments (e.g. Carey & Mayer 1990).

Since previous work has indicated differential sensitivity of oxic and anoxic microbial metabolism to bromophenols, we chose to compare the responses of aerobic ammonia oxidation with sulfate reduction. Ammonia oxidation plays a key role in nitrogen transformations, denitrification and benthic-pelagic coupling (e.g. Henriksen et al. 1981, Söderlund & Ross-wall 1982, Ward 1986, Henriksen & Kemp 1988, Blackburn & Sørensen 1988). Sulfate reduction in bulk sediments accounts for >50% of the total and up to 90% of the anaerobic organic matter mineralization in nearshore marine sediments, exceeding oxygen-linked carbon transformation rates in some cases (e.g. Jørgensen 1977, 1982, Canfield 1989, Canfield et al. 1993).

MATERIALS AND METHODS

Effect of bromophenols on sulfate reduction. Bulk sediments were collected from the lower intertidal region of Lowes Cove, Maine, USA (43° 56' 10" N, 69° 34' 30" W) at low tide using 6.5 cm diameter core tubes (see King 1988 and Hansen et al. 1996 for site descriptions). After returning to the laboratory, sediment cores were extruded and sectioned quickly in air. The 1 to 4 cm interval from 11 cores was pooled and homogenized by brief stirring in a nitrogen-filled glove-bag. Bromoorganic (BP, 2,4-DBP, 2,6-DBP and TBP) stock solutions at 25, 250 and 2500 μM were prepared in 0.2 μm filtered anoxic seawater, except for 2500 μM

TBP which was prepared in 0.1 M carbonate buffered seawater (Perrin & Dempsey 1974) due to its low solubility. Sediment and bromoorganic stock solutions were transferred to sealable plastic bags at a ratio of 3:2 (vol:vol) to produce final concentrations of 10, 100 and 1000 nmol cm^{-3} slurry. The solution and mud were thoroughly mixed by gentle kneading. The resulting slurries were dispensed into cut-off 5 cm^3 syringes through a port in the bags. Syringes were filled completely and immediately sealed with 20 mm butyl septa (Supelco, Inc.). Samples were incubated at 16°C under nitrogen.

Sulfate reduction rates for each treatment were determined using 5 sets of triplicate samples in a time series over an 11 d incubation period. 5 μCi (185 kBq) of carrier-free $^{35}\text{SO}_4^{2-}$ (New England Nuclear) prepared in 0.2 μm filtered seawater were injected uniformly along the length of the slurries through the butyl septa using a 10 μl syringe. After incubation at 16°C for 4 h, microbial activity was terminated by freezing the syringes at -80°C in a 95% ethanol bath. The frozen sediment samples were processed for total reduced inorganic sulfur by distillation in a solution of 1 M chromous chloride in 1 M HCl as described by King (1988). The efficiency of the distillation was determined using sodium sulfide standards (5 ml of a 160 mM solution). Sulfide recovery was measured spectrophotometrically according to King (1988). Recoveries were 95 to 98% with approximately 98% of the total sulfide being collected in the first zinc acetate trap.

Burrow wall sediments of *Protoglossus graveolens*, *Saccoglossus bromophenolosus* and *Mya arenaria* were collected from Lowes Cove while sediments of *S. kowalevskii* were collected from a site at the mouth of York River, Maine (43° 07' N, 70° 39' W). *Nereis virens* burrow wall sediments were collected from both sites. Burrow samples were obtained by exposing subsurface sediments and carefully scraping the inner 1 to 2 mm of burrow wall sediment. Samples were collected only from burrows actively occupied by the target species. Bulk sediments were collected from the same sites through the use of 6.5 cm diameter cores. The cores were separated into 1 to 4 and 4 to 10 cm intervals. The respective sediment samples were slurried using 0.2 μm filtered seawater and dispensed in triplicate into cut-off 5 cm^3 syringes as above. The samples were incubated at 16°C for 4 h and assayed for sulfate reduction rates as described previously.

Effect of bromophenols on ammonium oxidation. Bulk sediment samples were collected from the mouth of Lowes Cove as above. In the laboratory, the top 1 to 2 mm of sediment was sectioned from 5 cores, pooled and briefly homogenized by mixing. A slurry was prepared by adding 0.2 μm filtered seawater to the sediment at a ratio of 1:10 (sediment:seawater). Samples of

10 ml of this slurry were dispensed into 50 ml conical polystyrene centrifuge tubes. Four tubes were set aside as controls. BP, 2,4-DBP, 2,6-DBP and TBP, prepared as stock solutions of 20 mM in 0.1 M carbonate buffer (pH 10.1), were added to sediment slurries in triplicate at final concentrations of 1, 5, 10, 100 and 1000 nmol cm⁻³ slurry. Ammonium chloride and sodium chlorate, an inhibitor of nitrite oxidation (Belser & Mays 1980), were dispensed into each tube at final concentrations of 1 and 10 mM, respectively. All tubes were incubated horizontally on an orbital shaker (190 rpm) at 20°C for 5 to 6 d. Potential ammonium oxidation rates were determined by measuring the accumulation of nitrite (Grasshoff 1976). Subsamples (200 µl) were obtained regularly with a pipettor fitted with cut-off 250 µl tips. Nitrite levels were measured after centrifuging subsamples at 10500 × g in a microfuge for 10 min. Supernatant (100 µl) was pipetted into 900 µl of DI and mixed with 20 µl each of sulfanilamide and n-(1-naphthyl)ethylenediamine dihydrochloride (Grasshoff 1976). The absorbance of the reaction mixture was measured at 543 nm on a Beckman 640 spectrophotometer.

Burrow wall sediments of *Protoglossus graveolens*, *Saccoglossus bromophenolosus*, *Nereis virens* and *Mya arenaria* were collected as described above. However, care was taken to collect only the inner 1 mm of burrow wall sediment in volumes small enough to avoid anoxia during storage and transport. The sediment was immediately processed in the laboratory by making a homogeneous mixture of each burrow type. From each burrow sample 1 cm⁻³ of sediment was dispensed into 50 ml conical polystyrene centrifuge tubes (5 replicates per burrow type). The sediment in each tube was diluted 1:10 with 0.20 µm filtered seawater and amended with ammonium chloride and sodium chlorate as above. All treatments were incubated as above for a period of 5 d with subsamples collected and processed as before.

Bromophenol concentration in burrow wall sediments. *Protoglossus graveolens* and *Saccoglossus bromophenolosus* burrow wall samples were extracted with hexane and analysed by gas chromatography in order to determine the 2,4-DBP concentrations. Four replicates of 9 cm³ sediment for each burrow type were placed into 15 ml screwcap GC vials with 2 ml hexane; the vials were sealed with teflon-coated neoprene septa and the sediments extracted at room temperature for 24 h. Subsamples of 2 µl of the hexane were injected into a Varian 3400 gas chromatograph fitted with a J&W Scientific DB-5 column and operated as described by King et al. (1995).

Table 1. Relative sulfate reduction activity in sediments augmented with bromophenols (concentrations at 1 µmol cm⁻³) expressed as percent of rates observed in control treatments (mean ± SE, n = 3). *Time points where rates in treatments with bromophenols were significantly lower than control rates (ANOVA with Tukey's test at p < 0.05)

Treatment	Incubation period (h)		
	0	24	28
4-Bromophenol	71.9 ± 3.6*	90.4 ± 11.8	99.1 ± 4.4
2,4-Dibromophenol	11.4 ± 1.1*	11.0 ± 1.9*	39.6 ± 5.2*
2,6-Dibromophenol	68.4 ± 5.5*	59.0 ± 8.0*	44.3 ± 0.7*
2,4,6-Tribromophenol	30.3 ± 7.8*	36.4 ± 7.0*	88.0 ± 9.8

RESULTS

Effect of bromophenols on sulfate reduction

No significant differences (p < 0.05) relative to controls or among treatments were observed at 10 and 100 nmol cm⁻³ levels of bromophenols throughout the 10 d incubation period. Augmentation of bulk sediments with bromophenols at 1000 nmol cm⁻³ resulted in significant inhibition of sulfate reduction (Table 1). The pattern observed for 2,4-DBP was typical (Fig. 1). The duration of inhibition was shortest for BP with rates equalling those of controls within 24 h. 2,4-DBP and TBP showed the most dramatic levels of inhibition over the initial 24 h (Table 1); however, inhibition was most persistent for 2,6-DBP, with rates significantly below those of the controls for 48 h (Table 1).

Sulfate reduction rates in burrow wall sediments of *Saccoglossus bromophenolosus* from Lowes Cove were significantly lower than rates in bulk sediment from 1 to 4 cm depth, but did not differ significantly from rates at 4 to 10 cm (Fig. 2). An inverse trend was apparent in burrow wall sediments of *S. kowalevskii* from York River, with rates statistically equal to or

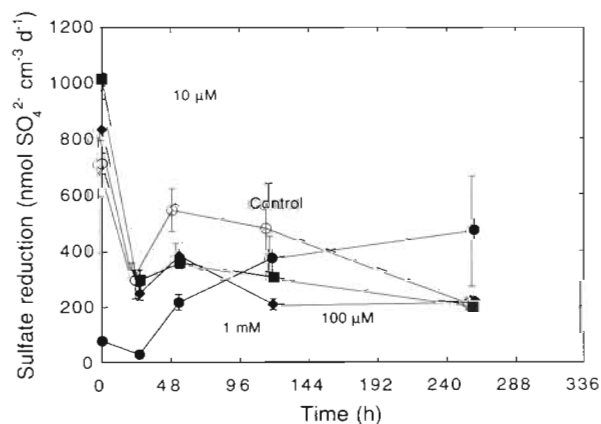


Fig. 1. Temporal pattern of sulfate reduction rates (mean ± SE, n = 3) in sediments augmented with 2,4-dibromophenol at 10 (■), 100 (◆) and 1000 (●) nmol cm⁻³. (○) Control

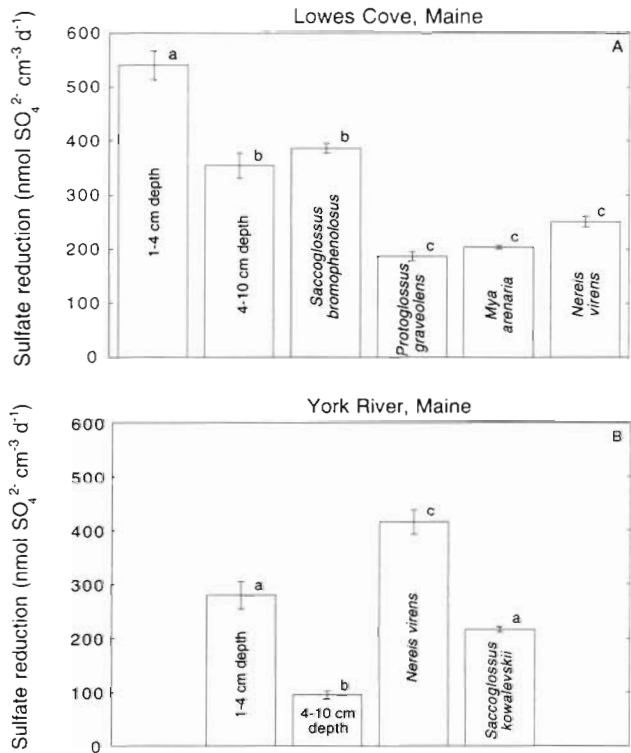


Fig. 2. Sulfate reduction rates (mean \pm SE, $n = 3$) in: (A) burrow wall sediments of *Saccoglossus bromophenolosus*, *Protoglossus graveolens*, *Nereis virens*, *Mya arenaria*, and surrounding sediments collected from Lowes Cove, Maine; (B) burrow wall sediments of *S. kowalevskii*, *N. virens*, and surrounding sediments collected from York River, Maine. Statistical analyses performed by ANOVA with Tukey's test at $p < 0.05$; different letters refer to significant differences at $p < 0.05$

greater than those in bulk sediments from 1 to 4 and 4 to 10 cm depth, respectively (Fig. 2). Sulfate reduction rates in burrow wall sediments of *Protoglossus graveolens*, *Mya arenaria* and *Nereis virens* collected from Lowes Cove were significantly lower ($p < 0.05$) than in bulk sediments from 1 to 4 and 4 to 10 cm; rates in burrow wall sediments among these taxa did not differ statistically (Fig. 2). In contrast, sulfate reduction rates for *N. virens* burrow wall sediments collected from York River were significantly greater ($p < 0.05$) than rates in bulk sediments and burrow wall sediments of *S. kowalevskii* (Fig. 2).

Table 2. Concentration of 2,4-dibromophenol in burrow wall sediments of 2 enteropneusts (mean \pm SE, $n = 4$)

Species	μM (fresh)	ng (g dry wt) $^{-1}$
<i>Protoglossus graveolens</i>	2.68 ± 0.61	600 ± 132.7
<i>Saccoglossus bromophenolosus</i>	0.16 ± 0.03	40 ± 8.1

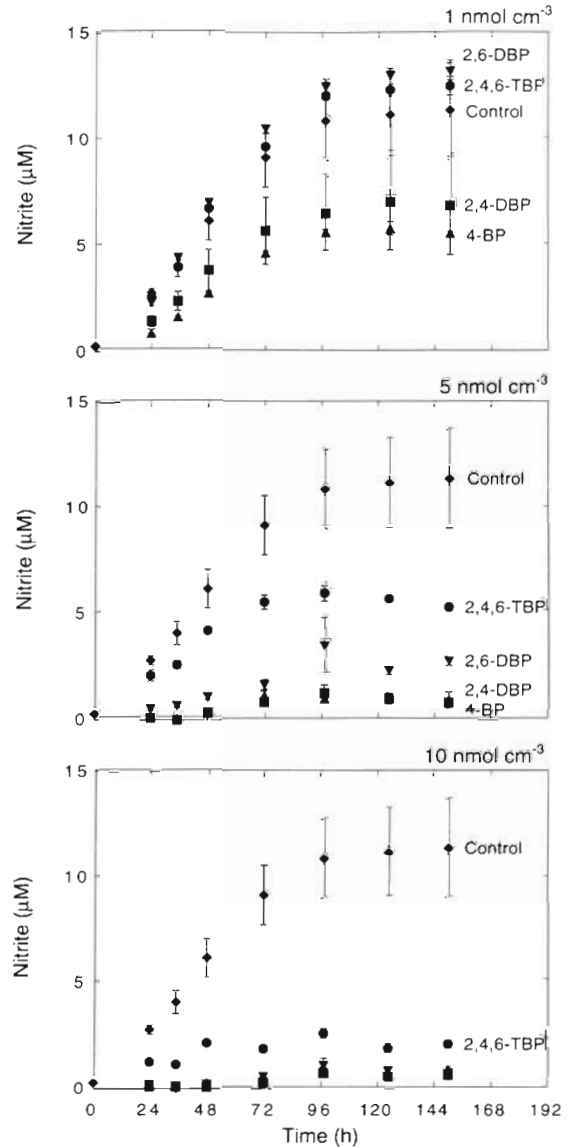


Fig. 3. Ammonium oxidation as nitrite accumulation (mean \pm SE, $n = 3$) in surface sediments augmented with bromophenols at 1, 5 and 10 μM

Gas chromatographic analysis revealed substantial 2,4-DBP concentrations in burrow wall sediments of *Protoglossus graveolens*. Concentrations were detectable, but an order of magnitude lower, in burrow wall sediments of *Saccoglossus bromophenolosus* (Table 2).

Effect of bromophenols on ammonium oxidation

Ammonium oxidation was completely inhibited by all 4 bromophenols at concentrations of 100 and 1000 nmol cm^{-3} (not shown). At 10 nmol cm^{-3} ammonium oxidation was completely inhibited by 2,4-DBP and strongly suppressed by the other bromophenols

in the following order: BP, 2,6-DBP and TBP (Fig. 3). Ammonium oxidation was also significantly inhibited in all treatments at 5 nmol cm⁻³, and with 2,4-DBP and BP at 1 nmol cm⁻³ (Fig. 3; $p < 0.05$). Nitrite levels in 2,6-DBP and TBP treatments at 1 nmol cm⁻³ did not differ significantly from the controls (Fig. 3).

Ammonium oxidation was enhanced in burrow wall sediments of *Nereis virens*, with nitrite production almost an order of magnitude higher than in surface sediment (Fig. 4). Although not quite as dramatic as in burrow wall sediments of *N. virens*, ammonium oxidation was also elevated in burrow wall sediments of *Mya arenaria* and *Saccoglossus bromophenolosus*, with peak nitrite concentrations 3- to 5-fold above those in surface sediments (Fig. 4). Based on the initial increase in nitrite, rates of ammonium oxidation were significantly higher ($p < 0.05$) in burrow wall sediments of *S. bromophenolosus* than those of *M. arenaria* (Fig. 4). However, nitrite levels were significantly depressed ($p < 0.05$) below those of surface sediments in treatments containing *Protoglossus graveolens* burrow wall sediment (Fig. 4). Ammonium oxidation in these sediments was also significantly lower ($p < 0.05$) than rates for all other burrow wall sediments (Fig. 4).

DISCUSSION

Observations on the effects of bromophenols on 2 predominant microbial processes in marine intertidal sediments indicate that an aerobic process, ammonium oxidation, is considerably more sensitive than an anaerobic process, sulfate reduction. Results show that the addition of 2,4-DBP to sediment at concentrations as low as 1 nmol cm⁻³ sediment inhibits ammonium oxidation whereas concentrations approaching 1000 nmol cm⁻³ are required to inhibit sulfate reduction. These results are consistent with earlier reports by King (1988) showing greater impacts of 2,4-DBP on aerobic bacteria, and the behavior of these compounds as potent uncouplers of oxidative phosphorylation (Stockdale & Selwyn 1971a, b). Antibiotic activity towards microbes and meiofauna (Azariah et al. 1978, King 1986, 1988, Weiss et al. 1986, Jensen et al. 1992, Teeyapant et al. 1993) and nephrotoxic and hepatotoxic activity in higher organisms (Kerger et al. 1988, Casillas & Myers 1989) have previously been documented for bromophenols and other brominated metabolites.

The inhibition pattern for sulfate reduction suggests that sulfate-reducing bacteria are sensitive to bromophenols, though not to the extent noted for aerobes.

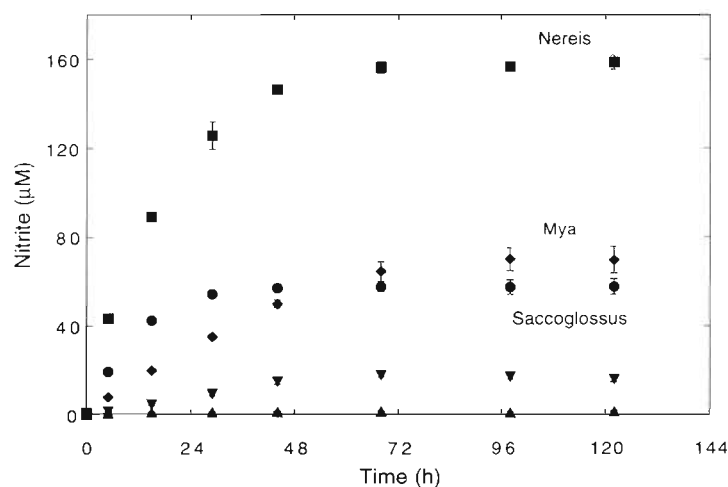


Fig. 4. Ammonium oxidation as nitrite accumulation (mean \pm SE, $n = 3$) in burrow wall sediments of *Saccoglossus bromophenolosus* (●), *Protoglossus graveolens* (▲), *Nereis virens* (■), *Mya arenaria* (◆), and surface sediments (▼) collected from Lowes Cove, Maine

The effect of bromophenols on sulfate reduction appears to be tempered by the rapid dehalogenation of bromophenols under anoxic conditions (e.g. King 1988, Steward et al. 1995). The pattern of 2,4-DBP degradation previously reported by King (1986, 1988) is consistent with the time course of inhibition reported here. Inhibition of sulfate reduction in bulk sediments occurs during the initial incubation period following amendment with bromophenols at 1000 nmol cm⁻³ sediment. However, sulfate reduction is not inhibited at 100 nmol cm⁻³ 2,4-DBP and 300 nmol cm⁻³ TBP at any time.

These results suggest that the rates of sulfate reduction are depressed for the period required for substantial dehalogenation of 2,4-DBP and TBP (Fig. 5,

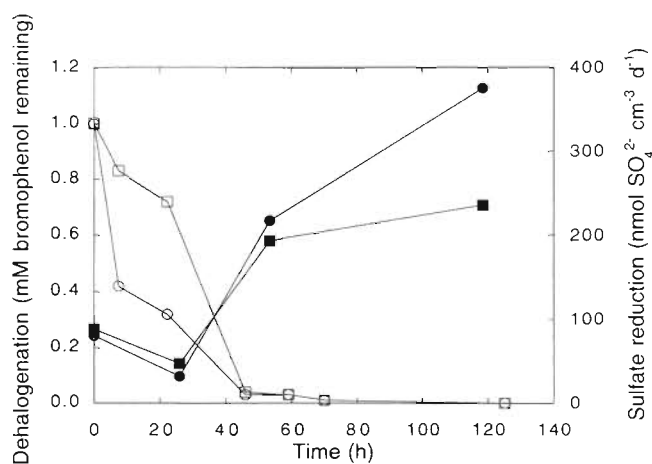


Fig. 5. Rates of sulfate reduction (solid symbols) and dehalogenation (from King 1988) compared for 1 mM concentrations of 2,4-dibromophenol (●, ○) and 2,4,6-tribromophenol (■, □)

Table 1). Inhibition during the first 24 to 48 h corresponds to the period during which bromophenols are dehalogenated to form phenol. Stimulation of sulfate reduction after 24 to 48 h is probably the result of the direct degradation of phenol by sulfate-reducing bacteria as reported by Bak & Widdel (1986), or of acetate formed from phenol. Although dehalogenation by sulfate reducers has been documented, molybdate does not inhibit 2,4-DBP degradation (King 1988). Thus, it is likely that bromophenol degradation depends on the sequential activities of distinct dehalogenating and fermentative populations as has been reported for chlorobenzoate degradation (Dolfing & Tiedje 1991, Fulthorpe et al. 1996, Townsend & Suflita 1996).

Although sulfate reduction in bulk sediments is affected by added bromophenols, burrow wall sediments of *Saccoglossus bromophenolosus* and *S. kowalevskii* show no consistent difference relative to untreated surrounding sediments. This suggests little or no impact on sulfate reduction of bromophenol (or bromopyrrole) excretion by enteropneusts *in situ*. The low sulfate reduction rates in *Protoglossus graveolens* burrow wall sediments are not likely to be the result of bromophenol excretion since sulfate reduction rates in burrow wall sediments of *Mya arenaria* and *Nereis virens*, which do not produce bromophenols, are similar to rates for *P. graveolens*. In addition, 2,4-DBP concentrations in *P. graveolens* burrow sediments (Table 2) are far below levels that inhibit sulfate reduction *in vitro* (Fig. 1).

Bromophenols inhibit potential rates of ammonium oxidation in bulk surface sediments at concentrations nearly 1000-fold lower than those that inhibit sulfate reduction (Fig. 3). The inhibition of ammonium oxidation also persists much longer than inhibition of sulfate reduction. The greater persistence of inhibition under oxic conditions is undoubtedly due to the relatively slow rate of oxic bromophenol degradation (King 1986, 1988).

In this study, rates of ammonium oxidation are enhanced in burrow wall sediments relative to bulk surface sediments with the degree of enhancement decreasing in the following order: *Nereis virens*, *Saccoglossus bromophenolosus*, *Mya arenaria* (Fig. 4). In contrast, ammonium oxidation appears to be inhibited in burrow wall sediments of *Protoglossus graveolens* (Fig. 4). The variation in rates among *N. virens*, *S. bromophenolosus*, and *M. arenaria* may be due to differences in the rate of burrow ventilation (Foster-Smith 1978, Kristensen et al. 1991) or organic coatings on the burrow wall (Aller et al. 1983, Kristensen et al. 1985).

However, 2,4-DBP concentrations in burrow wall sediments of *Protoglossus graveolens* (Table 2) are sufficient to inhibit ammonium oxidation (Figs. 3 & 4). Thus, ammonium oxidation, unlike sulfate reduction, may be adversely affected by 2,4-DBP in *P. graveolens* burrows. Nonetheless, it seems unlikely that any effects

on *P. graveolens* burrows would be manifest through changes in benthic nitrogen fluxes, except in cases where *P. graveolens* dominates the benthic fauna.

Differences between *Saccoglossus bromophenolosus* and *Protoglossus graveolens* in both burrow wall ammonium oxidation (Fig. 4) and 2,4-DBP concentrations (Table 2) may be attributed to lower rates of 2,4-DBP excretion by *S. bromophenolosus* or more rapid 2,4-DBP degradation in its burrow. At present, there is insufficient information to distinguish between the 2 explanations. However, the generally rapid turnover of 2,4-DBP in bulk sediments indicates that differences in excretion are most likely.

In general, our results suggest that aerobic microbial processes, such as ammonium oxidation, can be affected by naturally occurring bromophenols at ambient concentrations in burrow wall sediments. Although ammonium oxidation appears especially sensitive to bromophenol inhibition, other aerobic processes, including thymidine uptake, may be relatively insensitive (Steward et al. 1992, 1996). Likewise, anaerobic processes such as sulfate reduction appear insensitive to bromophenols, at least at typical ambient concentrations. Our results also suggest that bromophenol isomers differ in their potency as inhibitors. These differences, along with intrinsic differences in bromophenol excretion, must be considered in comparisons of burrow wall biogeochemistry among the bromophenol-containing benthic fauna.

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