Limnol. Oceanogr., 49(3), 2004, 716–726

Oxidative coupling during gut passage in marine deposit-feeding invertebrates

Anders M. B. Giessing[1] *and Lawrence M. Mayer*
Darling Marine Center, University of Maine, Walpole, Maine 04573

Abstract

We tested for oxidative coupling of phenolic materials, including organic contaminants and tyrosine, in gut fluid from marine deposit-feeding invertebrates. A phenolic metabolite (pyrenol) of a polycyclic aromatic hydrocarbon (PAH), which can arise during accumulation by deposit-feeding invertebrates, was found to participate in oxidative coupling reactions to organic matter in gut fluid. Gut fluid from four species of marine deposit-feeding invertebrates (three polychaetes and one holothuroid) catalyzed oxidative coupling of pyrenol (1-hydroxypyrene) in rough proportion to their enzyme activity and dissolved organic matter levels. *Nereis virens* gut fluid also catalyzed coupling of tyrosine monomers to form dityrosine, a common marker of oxidative damage in proteins. An antioxidant enzyme activity, similar to that of heme peroxidase and capable of oxidative coupling, was tentatively identified in gut fluid from *N. virens* by protein precipitation, dye decolorization assays, and enzyme inhibitor studies. Unaltered *N. virens* gut fluid had high total oxyradical scavenging capacity, indicating the presence of fast-acting antioxidants. Oxidative coupling of PAHs will reduce subsequent bioavailability, toxicity, and transport of these compounds in marine environments. Furthermore, oxidative coupling of PAHs represents a hitherto overlooked sink for organic contaminants in marine sediments and suggests a biological mechanism for formation of aquatic humic material.

Deposit-feeding invertebrates dominate metazoan abundance and biomass in marine sediments (Pearson and Rosenberg 1978; Lopez and Levinton 1987). These animals strongly influence sediment ecosystems by their vertical and horizontal mixing activities (Levinton 1995; Aller and Aller 1998; Aller et al. 2001) and perhaps via direct reactions during gut passage.

The low concentration and refractory nature of sedimentary organic matter implies that deposit feeders survive on a remarkably poor food source (Lopez and Levinton 1987; Jumars and Wheatcroft 1989). Deposit feeders meet their nutritional requirements by ingesting large amounts of bulk sediment; for example, the lugworm (*Arenicola marina*, Polychaeta) ingests up to 20 times its body weight of wet sediment per day (Cadée 1976). Deposit-feeding metazoans have evolved an enclosed extracellular digestive geometry, which in some species lets them efficiently retain both digestive agents and products, building up high concentrations of enzymes, surfactants, and other dissolved organic matter (DOM) in the midgut region (Mayer et al. 2001*a*).

Deposit feeders guts thus have very different chemistry than ambient sediment, with potential consequences that are ancillary to the digestion of sedimentary food. For example, high concentrations of dissolved transition metals, especially manganese and iron, are found in guts (Chen et al. 2000). These metals can be redox active and catalyze the generation of highly reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) (Winston and Digiulio 1991; Felton 1995). Extracellular ROS also form as a consequence of normal environmental processes. Intertidal invertebrates are not only exposed to varying concentrations of O_2 capable of inducing oxidative stress, but also high concentrations of hydrogen peroxide (H_2O_2) generated by ultraviolet radiation (Abele-Oeschager et al. 1994; Buchner et al. 1996). Sediments also contain significant amounts of photooxidation and other decay products of senescent phytoplankton cells, including hydroperoxides (Rontani and Marchand 2000; Marchand and Rontani 2001). Production of ROS can also be increased by uptake of polycyclic aromatic hydrocarbons (PAHs) and their photooxidation products (Livingstone et al. 1990; Arfsten et al. 1996).

High ROS and other free radical species may be stressful. Because of the instability and reactivity of ROS and their potential to damage cells and tissue, both antioxidant enzymes (e.g., superoxide dismutase, glutathione peroxidases, and catalases) and low–molecular mass antioxidants (e.g., glutathione, vitamins E and C, and carotenoids) are often made by organisms to protect against their adverse effects (Larson 1997). Although little is known about the presence and function of extracellular antioxidant enzymes in aquatic invertebrates, high activities of cytosolic antioxidant enzymes have been reported in chloragog digestive tissue of deposit-feeding polychaetes (Buchner et al. 1996).

Of special interest among the antioxidant enzymes is peroxidase, from the class of oxidoreductases. This type of enzyme can catalyze the formation of humic substances in soil via oxidative coupling reactions (Bollag et al. 1998) that start by generating free radicals in the reduction of a peroxide to water. The free radicals can undergo coupling, producing carbon–carbon and carbon–oxygen cross-linked polymers, or react with electron-rich biomolecules such as polyunsaturat-

[1] To whom correspondence should be addressed. Present address: Danish Institute for Food and Veterinary Research, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark (anders@giessing.dk).

Acknowledgments

This study was made possible in part by a stipend from The Danish Natural Science Research Council, a Fulbright Scholarship to A.M.B.G., the Agatha B. Darling Professor endowment, and the National Science Foundation (Chemical Oceanography). Additional financial support to A.M.B.G. was obtained through the COWI Foundation and the Torben and Alice Frimodts Foundation. Special thanks are extended to Rikke Hansen and Linda Schick for field and laboratory assistance and to I. Voparil, D. Weston, and R. Self for gut fluid samples. Thanks are also extended to two anonymous reviewers for their thorough and constructive review of the manuscript. Contribution number 389 from the Darling Marine Center.

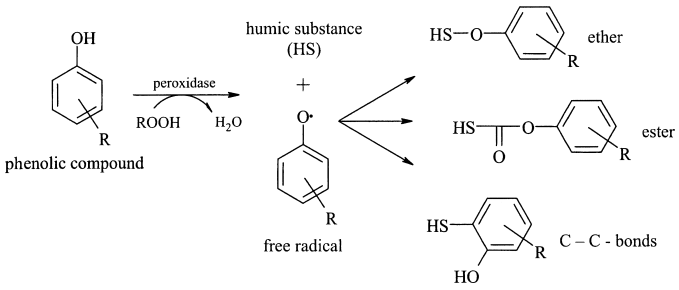

Fig. 1. Oxidative coupling of a phenolic compound to a model humic substance catalyzed by a peroxidase. Phenolic reactants are linked through C-O or C-C bonds in a free radical process.

ed fatty acids, proteins, and nucleic acids, creating large molecules with complex structures (i.e., humic substances; Fig. 1). Structural studies of aquatic humic matter have shown that precursor constituents containing carbon–carbon double bonds are important in the formation of humic acids from microbial and algal biomass; possible precursors are certain lipids, amino acids, and pigments (McKnight and Aiken 1998). Oxidative coupling processes can also be catalyzed abiotically by oxides of Al, Fe, and Mn, as well as clay minerals (Shindo and Huang 1984; Filip and Preusse 1985; Naidja et al. 2000), and oxidative coupling of phenols by MnO_2 has been recognized as important in formation of aquatic humic substances (Larson and Hufnal 1980).

Because degradation products of many pollutants (e.g., polycyclic aromatic hydrocarbons [PAHs]) also have phenolic characteristics, they can thus be incorporated into organic matter (Richnow et al. 1994, 1995; Weber and Huang 2003). Oxidative coupling of pollutants to natural macromolecular organic matter is believed to decrease bioavailability, toxicity, and transport in natural environments (Bollag 1992) and is believed to be a major sink for organic pollutants in soils (Bollag 1983). The gut of a marine deposit feeder is assumed to the primary route of both uptake and elimination of organic contaminants (Forbes et al. 1996; Mayer et al. 1996). We have recently identified pyrenol (1-hydroxypyrene), a phenolic metabolite of the tetracyclic PAH pyrene, in gut fluid of the deposit-feeding polychaete *Nereis diversicolor* (Giessing et al. 2003). Pyrenol's presence in gut fluid provides a phenolic substrate capable of participating in oxidative coupling reactions. Oxidative coupling of PAH metabolites during gut passage introduces a novel fate for organic contaminants in marine sediments.

This study investigated oxidative coupling reactions of phenolic compounds in gut fluid of marine deposit-feeding invertebrates. The study is part of an ongoing effort to study the effect of deposit feeder gut chemistry on the fate of organic contaminants, as well as natural organic matter in marine sediments. Our identification of pyrenol in gut fluid makes it a suitable model substrate for studying oxidative coupling reactions. Pyrenol has known oxidative coupling potential (Richnow et al. 1997), and both pyrenol and pyrene are readily measured at concentrations below aqueous solubility. Oxidative coupling of pyrenol implies formation of a pyrenolate-free radical with subsequent formation of cross-coupling products such as a pyrenol dimer. Richnow et al. (1997) found that cross-linking of a phenolic PAH was insignificant in the presence of humic substances. Deposit feeder gut fluid contains high concentrations of organic matter, with which a pyrenolate radical can react, making identification of an oxidative coupling product unlikely. Thus, for product studies, a more abundant naturally occurring compound, with aqueous solubility orders of magnitude higher than pyrenol, was needed. Tyrosine, an essential amino acid with phenolic functionality, was chosen for product identification experiments. A typical feature of oxidative stress in the physiological pH range is the formation of tyrosyl radicals by ROS. Protein-bound dityrosine, a major product of tyrosine modification, forms through a carbon–carbon bond between two tyrosine radicals (Jacob et al. 1996; Pennathur et al. 1999) and leads to molecular cross-links, which initiate dysfunction and aggregation. Identification of dityrosine formation catalyzed by gut fluid can provide evidence for oxidative coupling reactions of naturally occurring compounds during gut passage in marine deposit-feeding invertebrates.

An assay for oxidative coupling reactions was developed with the use of gut fluid from the omnivorous marine polychaete *Nereis virens.* The assay was applied to gut fluid from three other species of deposit-feeding invertebrates, *Arenicola brasiliensis* and *A. marina* (Annelida: Polychaeta) and *Parastichopus californicus* (Echinodermata: Holothuroidea). The ability of a biological fluid to scavenge ROS can be used as a measure of its antioxidant capacity without identifying the individual antioxidant agents (Winston et al. 1998). Because deposit feeder gut fluid is a complex soup of proteins, lipids, and metals, involvement of peroxidase-like enzymes in oxidative coupling reactions was studied by measuring the total oxyradical scavenging capacity (i.e., the total antioxidant capacity) of unaltered gut fluid, by protein precipitation experiments, and by dye decolorization assays and enzyme inhibitor studies.

Materials

Horseradish peroxidase (EC 1.11.1.7), Remazol Brilliant Blue R (RBBR), α-keto-γ-methiolbutyric acid (KMBA), and trichloroacetic acid (TCA) were obtained from Sigma. Pyrene (98%), pyrenol (98%), reduced glutathione (98%), 2,2′-azobis-amidinopropane (ABAP), and $Na_2S_2O_5$ was purchased from Aldrich Chemical Company. Hydrogen peroxide (30%) and NaCN were purchased from Fisher Scientific. L-(+)-Cysteine, Na_2HPO_4 (>99%), NaOH, and HCl were obtained from J. T. Baker. Acetonitrile, methanol, hexane, dichloromethane, and acetone, all ChromAR® high-performance liquid chromatography (HPLC) grade, were purchased from Mallinckrodt. Ethanol (100%) was obtained from Quantum Chemical Corporation. All chemicals were used as received.

PAH stock solutions were prepared by dissolving pyrene and pyrenol in methanol and adding 1 ml methanol stock to 1 liters of 0.02 mol L^{-1} Na_2HPO_4 buffer, adjusted with HCl to pH 7, to give final concentrations of 129 and 134 μg L^{-1}, respectively. Stock concentrations were based on an aqueous pyrene solubility of 0.64 μmol L^{-1} (Meyerhoffer and

McGown 1991). To our knowledge, there exist no data on aqueous solubility of pyrenol, but the addition of an O-containing functional group implies that pyrenol is more soluble than pyrene.

Test animals—All analytical assays were developed with the use of gut fluid from the marine polychaete *N. virens*, which were purchased from a commercial worm supplier in Edgecomb, Maine. Its digestive fluid was carefully extracted as described in Mayer et al. (2001*b*). Animals were held in seawater and dissected within 24 h after collection. Dissection and digestive fluid removal were performed under air. We avoided contamination with seawater by making a small insertion in the outer body wall to expose the gut, inserting a pipette through the gut wall, and withdrawing digestive fluid. Fluid from 80 individuals was pooled and centrifuged for 5 min at 420 × *g*, filtered (0.22 μm) and stored at −80°C prior to use. Gut fluid used in the experiments was always filtered through a 0.22-μm syringe filter unless otherwise stated. Gut fluid from the three other species, extracted in a similar manner, was taken from our stock of digestive fluids stored in 1-ml aliquots at −80°C. *A. marina* originated from sand flats at Lubec, Maine (courtesy of I. Voparil); *A. brasiliensis* was from San Francisco, California (courtesy of D. Weston); and *P. californicus* was from Puget Sound, Washington (courtesy of L. Self).

Methods

Oxidative coupling of pyrenol is, for lack of identification of an oxidative coupling product, defined as loss of pyrenol from aqueous solution when incubated with gut fluid and activated with H_2O_2. Pyrene is used as a nonreactive substrate with an aqueous solubility equal to pyrenol. Involvement of enzymes in the proposed oxidative coupling process was studied by measuring gut fluid total oxyradical scavenging capacity (TOSC) and ability to decolorize a dye.

Gut fluid assays with H_2O_2 activation—An oxidative coupling assay was developed with horseradish peroxidase (HRP). The fluorescence intensity of aqueous reaction mixtures containing 1 enzyme unit ml^{-1} HRP and 0.1 mg L^{-1} pyrenol or pyrene were recorded before and after five consecutive additions of 1 mmol H_2O_2. The fluorescence intensity of pyrenol at excitation/emission wavelengths ($\lambda_{EX/EM}$) = 350/384 nm fell immediately below detection on addition of H_2O_2, whereas pyrene fluorescence ($\lambda_{EX/EM}$ = 338/372 nm) was lowered by only 2% after each addition of H_2O_2 (data not shown). The significant loss of pyrenol fluorescence indicates effective binding of pyrenol to itself or to other reactants in the sample matrix. The fluorescence signal was not affected by addition of either deionized water (DI) instead of H_2O_2 or H_2O_2 without HRP (data not shown).

The *N. virens* gut fluid assay was identical to the HRP assay described above except that 30 μl of 0.22-μm filtered gut fluid was substituted for HRP and added to 1.5 ml PAH stock solution. To reduce quenching of the pyrenol fluorescence signal, gut fluid was diluted 100-fold, and all experiments were performed with an Instech cuvette stirrer (Instech Laboratories, Inc.) during data collection. H_2O_2 (3%, 15 μl) was added, and the fluorescence signal of pyrenol ($\lambda_{EX/EM}$ = 350/384 nm) and pyrene ($\lambda_{EX/EM}$ = 338/372 nm) was recorded over time. Although the method worked well for qualitative studies, the need for continuous stirring of the cuvette proved cumbersome, and a cleanup and chromatographic separation step was included.

Oxidative coupling potential of gut fluid was tested by adding 100 μl undiluted gut fluid to 1 ml of PAH stock in 1.5-ml Eppendorf tubes and initiating the reaction by addition of 50 μl of 0.1 mol L^{-1} H_2O_2. The nonactivated control was a sample with 50 μl DI added instead of H_2O_2. Triplicate samples were placed in an incubator oven-mounted on an orbital shaker at 37°C for 1 h. After incubation, 0.5 ml of the sample was transferred to a clean Eppendorf tube, and 0.5 ml of ice-cold 100% ethanol or 10% (v/v) trichloroacetic acid (TCA; *see* Mayer et al. 1995) was added to precipitate high–molecular weight (HMW) protein. Precipitation of HMW protein was used to denature protein and stop the enzymatic reaction and as a sample cleanup procedure prior to chromatographic analysis. The samples were mixed and centrifuged at 420 × *g* at 0°C for 5 min, and 0.5 ml of the supernatant was transferred directly to brown HPLC vials without further preparation or extraction. Gut fluids from three other species (*A. brasiliensis, A. marina,* and *P. californicus*) were incubated and analyzed as described above. Analysis of dissolved pyrene and pyrenol was done by HPLC with fluorescence detection (HPLC/F, *see below*).

Gut fluid assay without H_2O_2 activation—To test for oxidative capacity without H_2O_2 activation *N. virens* gut fluid incubations were followed over time. We added 1 ml of gut fluid to 10 ml of PAH stock in glass and left it in the dark at room temperature. The reaction mixture was subsampled in duplicate (2 × 100 μl) and prepared for HPLC analysis as in the first experiment. As a control, 250 μmol L^{-1} sodium metabisulfite ($Na_2S_2O_5$) was added to inhibit the hypothesized oxidative process.

Tyrosine experiment—Because dityrosine is not commercially available, it was synthesized by oxidation of L-tyrosine with HRP modified after Malencik et al. (1996). To a 5 mmol L^{-1} L-tyrosine solution was added 10 mg HRP (116 enzyme units mg^{-1}) and 1.25 mmol H_2O_2 (0.5 mol H_2O_2 mol^{-1} tyrosine) and briefly swirled. The reaction was stopped after 20 min by addition of 175 μl β-mercaptoethanol to the reaction mixture. The golden brown solution was lyophilized and then dissolved in HPLC buffer. HRP was separated by centrifugation in Microsep centrifugation tubes (Pall Life Sciences, 30.000 MW) at 5,000 × *g* for 30 min before injection on HPLC. Analysis of synthesized oxidation products was by reversed-phase HPLC (*described below*).

Formation of L-tyrosine oxidation products by *N. virens* gut fluid was studied by incubating 3 ml of 0.5 mmol L^{-1} L-tyrosine in 0.1 mol L^{-1} Na_2HPO_4 buffer (pH 8) with 150 μl gut fluid in the dark for 3 h. The reaction mixture was then centrifuged twice through Ultrafree-CL centrifugal filters (Millipore), with 30.000 MW and 10.000 MW cutoff, respectively, for 30 min at 5,000 × *g*. The final filtrate was diluted 1 : 10 with HPLC buffer and analyzed by HPLC/F as described for the dityrosine standard.

Total oxyradical scavenging capacity assay—One approach in assessing total antioxidant capacity of a biological fluid without isolating the individual radical scavengers is to measure its capability to absorb ROS by determining its total oxyradical scavenging capacity (TOSC) (Winston et al. 1998). Peroxyl radicals generated by thermal homolysis of 2,2′-azobis-amidinopropane (ABAP) cause the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene. In the presence of an antioxidant (in this case gut fluid), the oxidation of KMBA to ethylene is quantitatively reduced. Ethylene formation is then monitored by gas chromatography with flame ionization detection (GC-FID) of headspace gas in the reaction vessel. Reaction mixtures consisted of 0.20 mmol L^{-1} KMBA and 50 μl diluted gut fluid mixed in a 10-ml test tube to a final volume of 1 ml. Gut fluid dilutions were 1 : 50, 1 : 500, 1 : 750, and 1 : 1,000, as indicated in the legend of Fig. 7. The test tube was sealed with a rubber septum, and 50 μl of 0.4 mol L^{-1} ABAP was injected. The reaction mixture was allowed to equilibrate at 35°C in a thermostated waterbath prior to addition of ABAP. A headspace sample was drawn immediately after addition of ABAP with a 1-ml syringe and a Hamilton GC needle and analyzed on a Shimadzu GC-14A/FID with a 30-m Megabore DB-1 column (J&W Scientific). Sample volume was 300 μl. All reactions were carried out at 35°C because at this temperature, peroxyl radicals are generated at a constant rate from ABAP (1.66×10^{-8} mol L^{-1} s^{-1}). Headspace samples were then drawn every 10 min for 60 min. Reduced glutathione was used as a reference antioxidant at concentrations of 50, 100, and 150 μmol L^{-1}.

On the basis of the difference in ethylene formation between control (KMBA solution without gut fluid) and samples containing gut fluid, TOSC values were quantified according to the equation TOSC = $100 - (\int SA/\int CA \times 100)$/gTAA, where $\int$ SA and $\int$ CA are the numerically integrated areas under the kinetic curves of sample (SA) and control (CA) reactions, respectively, and gTAA is total amino acid content of the gut fluid. Areas were calculated by Kaleidagraph™ (Synergy Software).

RBBR decolorization assay—H_2O_2-dependent decolorization of certain dyes can indicate oxidoreductive enzyme activity; for example, RBBR has been used for peroxidases in fungi (Glenn and Gold 1983; Pasti and Crawford 1991; Vyas and Molitoris 1995). We tested small volumes of gut fluid for peroxidase-like activity by incubating a RBBR solution and activating it with H_2O_2. Initial experiments showed that RBBR decolorization depends on initial concentration of H_2O_2 (10–100 μmol L^{-1}) and the pH (6–8) of the reaction mixture. Highest reaction potential was observed at pH 8, with an initial H_2O_2 concentration of 100 μmol L^{-1} (data not shown), which was used in subsequent RBBR experiments. Reaction mixtures consisted of 2 ml of 0.1 mmol L^{-1} RBBR in 0.1 mol L^{-1} phosphate buffer. To all reaction mixtures was added 20 μl of *N. virens* gut fluid, and the initial absorbance at 595 nm was recorded. Controls consisted of RBBR with only gut fluid or H_2O_2 added. Known inhibitors of oxidative enzymes (Saunders et al. 1964; Dunford 1999) were tested by adding NaCN, $Na_2S_2O_5$, and cysteine as aqueous solutions to the reaction mixture after addition of gut fluid and prior to the addition of H_2O_2. Decolorization of RBBR was performed in polystyrene cuvettes (VWR) and a Hitachi 3010 spectrophotometer (slit width of 2 nm). Inhibition was calculated as $(A_i/A_0 - 1) \times 100$, where A_i is the absorption at 595 nm at different concentrations of inhibitor and A_0 is the absorption at 595 nm prior to addition of inhibitor.

Total amino acid analysis—Total amino acid (TAA) contents of gut fluids were analyzed according to Mayer et al. (1995). Gut fluid (10 μl; 100 μl for *P. californicus*) was first hydrolyzed in 6 N HCl at 110°C for 22 h, and the hydrolyzed samples were derivatized with orthophthaldialdehyde followed by fluorometric detection.

Spectroscopy and HPLC—In the HRP assay, pyrenol and pyrene were analyzed by fluorescence spectroscopy. At a constant wavelength difference of 34 nm, pyrene and pyrenol spectra are reduced to single peaks at $\lambda_{EX/EM}$ of 338/372 nm and 350/384 nm, respectively. We used methyl acrylate cuvettes in a Hitachi F-4500 spectrofluorometer, with 2.5/5 nm slit widths (emission/excitation), and a response time of 0.1 s.

In gut fluid experiments, pyrene and pyrenol were analyzed on a Hitachi D-7000 HPLC system by injection of aqueous supernatants on a Supelcosil LC-PAH column (250 $\times$ 4.6 mm, 5-μm particles, Supelco). The acetonitrile/water gradient profile was 60/40 (v/v) for 5 min, directly increased to 100/0 over 30 min, and then held at 100/0 for 10 min, all at flow rate = 1.5 ml min^{-1}. Fluorescence detection was performed at $\lambda_{EX/EM}$ = 346/384 nm for pyrenol and $\lambda_{EX/EM}$ = 333/384 nm for pyrene. Injection volume was 250 μl, and column temperature was kept at 30°C.

HPLC analysis of tyrosine oxidation products was done on a Beckman Ultrasphere ODS column (250 $\times$ 4.6 mm, 5-μm particles). Oxidation products were eluted isocratically at 1.8 ml min^{-1} with a mobile phase of 0.1 mol L^{-1} Na_2HPO_4 and 0.1 mmol L^{-1} edatic acid and adjusted to pH 5.9 with acetic acid. L-Tyrosine oxidation products were detected by fluorescence detection with $\lambda_{EX/EM}$ = 280/410 nm.

Results

Pyrenol uptake—Oxidative coupling reactions will affect fluorescence characteristics of pyrenol, resulting in loss of the original peak at $\lambda_{EX/EM}$ = 350/384 nm. *N. virens* gut fluid catalyzed a significant reduction in pyrenol fluorescence intensity at $\lambda_{EX/EM}$ = 350/384 nm when activated with 1 μmol H_2O_2 (Fig. 2). Fluorescence decreased immediately after H_2O_2 addition to pyrenol and gut fluid mixtures. Two controls containing pyrenol/gut fluid and pyrenol/H_2O_2 showed no similar decrease. An identical experiment with pyrene showed no decrease of pyrene fluorescence in any of the three treatments (data not shown).

We have defined oxidative coupling potential as the decrease in recovery of PAH from an aqueous solution after incubation with invertebrate gut fluid activated with H_2O_2. *N. virens* gut fluid activated with H_2O_2 reduced dissolved pyrenol to <0.5% of the concentration measured in the control experiment (Fig. 3) but showed no significant oxidative

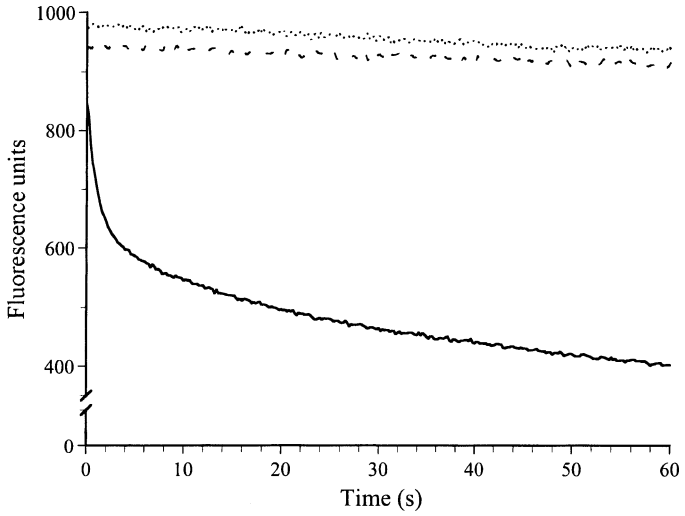

Fig. 2. Fluorescence time scan of an aqueous pyrenol stock solution with the addition of either *Nereis virens* gut fluid (dashed), 10 μl of 0.1 mol L^{-1} H_2O_2 (dotted line), or both (solid line). Fluorescence was recorded at $\lambda_{EX/EM}$ 350/384 nm with continuous stirring of the cuvette. Gut fluid was diluted 1 : 100 to reduce pyrenol quenching.

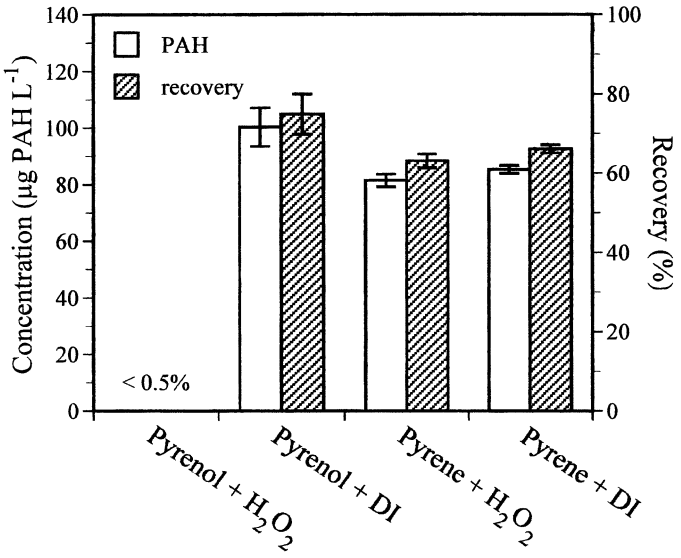

Fig. 3. Residual aqueous PAH in 10 : 1 mixtures of PAH stock and gut fluid from *Nereis virens* after incubation with 5 μmol H_2O_2. Data are presented as average ± SD (n = 3).

coupling potential with pyrene. Pyrene recoveries were not significantly different (t-test, $P > 0.05$) at 63% ± 2 and 66% ± 1 for H_2O_2-activated and control, respectively. An extraction test showed that recovery of pyrene with the centrifugation step omitted was slightly higher (87% ± 7, t-test, $P > 0.05$, n = 3), indicating some coprecipitation of pyrene with the protein. Pyrene recovery remained stable throughout the experiment, and substituting TCA for ethanol in the HMW protein preparation step had no effect on recovery of either pyrenol or pyrene (data not shown). Furthermore, pyrenol and pyrene concentrations of samples prepared using either precipitation method was stable for up to 72 h. Thus, lower than expected recoveries are most likely from uptake of PAH by gut fluid DOM.

N. virens gut fluid lowered the dissolved pyrenol concentration without addition of H_2O_2 (Fig. 4) to less than 10% after 2 h. Adding the oxidative inhibitor $Na_2S_2O_5$ reduced both the apparent rate and the final magnitude of removal by 63%. Pyrene concentration declined insignificantly over the experimental period.

Gut fluid from all four species tested showed significant pyrenol oxidative coupling potential when incubated with H_2O_2 (Fig. 5). Pyrenol recoveries were <0.5% in the polychaetes and 48% in the gut fluid from *P. californicus*. Recovery of pyrenol from DI controls (i.e., H_2O_2 nonactivated) varied between 53% and 93% among the four species. TAA contents of the gut fluid from the 4 species were 0.6, 52.5, 21.7, and 41.3 mg ml^{-1} for *P. californicus*, *A. marina*, *N. virens*, and *A. brasiliensis*, respectively. There was moderate, but not significant, negative correlation (Pearson product–moment correlation, P = 0.15) between the amount of TAA in the gut fluid and the average recovery of pyrenol. Thus, with the caveat of non–H_2O_2-activated pyrenol loss, we hypothesize that the variation in pyrenol recovery can, to some extent, be explained by uptake and subsequent coprecipitation with protein and other dissolved organic matter during sample preparation.

Dityrosine formation—L-Tyrosine was oxidized to dimers and trimers when incubated with gut fluid without the addition of H_2O_2 (Fig. 6). Three fluorescent peaks in gut fluid–

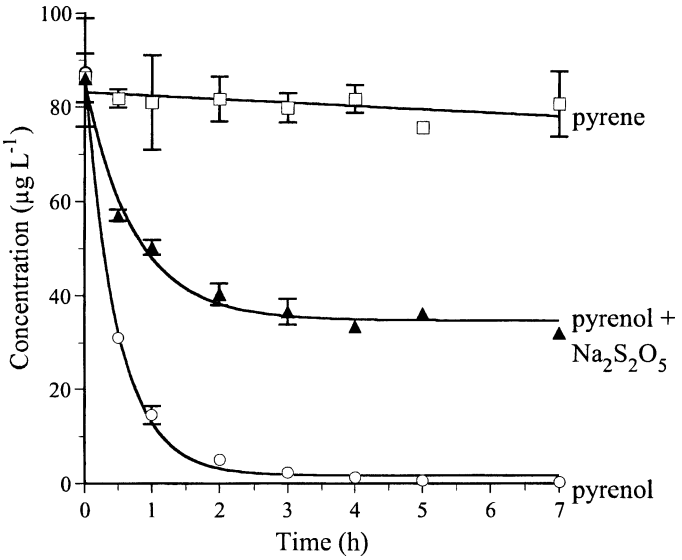

Fig. 4. Residual aqueous pyrenol in 10 : 1 mixtures of PAH stock and *Nereis virens* gut fluid in the absence of external H_2O_2. The concentration of dissolved pyrenol (open circles) is reduced to <10% of initial aqueous concentration within 2 h of incubation. Adding an oxidative enzyme inhibitor (250 μmol L^{-1} $Na_2S_2O_5$) to the assay (filled triangles) increases pyrenol recovery, whereas pyrene concentration (open squares) remains constant over the experimental period. Error bars (±1 SD) are given when their size exceeds that of the symbol used.

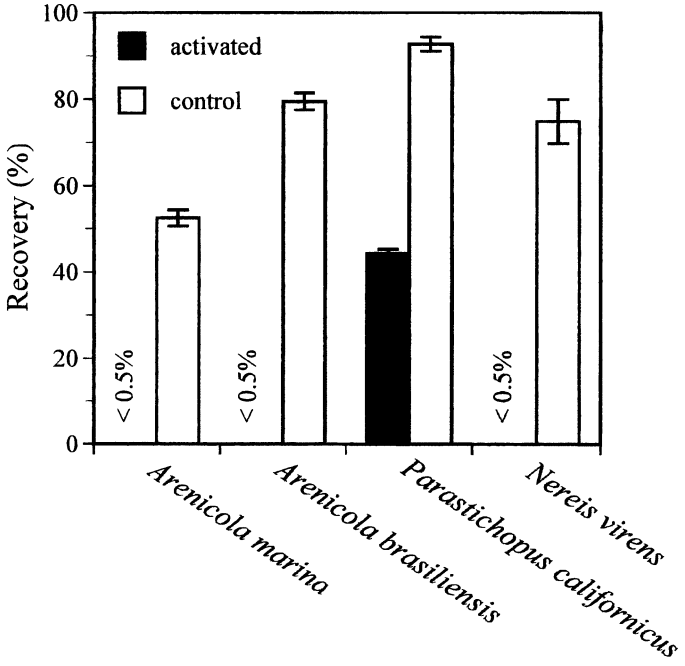

Fig. 5. Recovery of aqueous pyrenol in 10 : 1 mixtures of PAH stock and gut fluid from four deposit-feeding invertebrates incubated with 5 μmol H_2O_2. Black (H_2O_2-activated) and white (DI control) bars are recovery (%) relative to initial pyrenol concentration. Data are presented as average ± SD (n = 3).

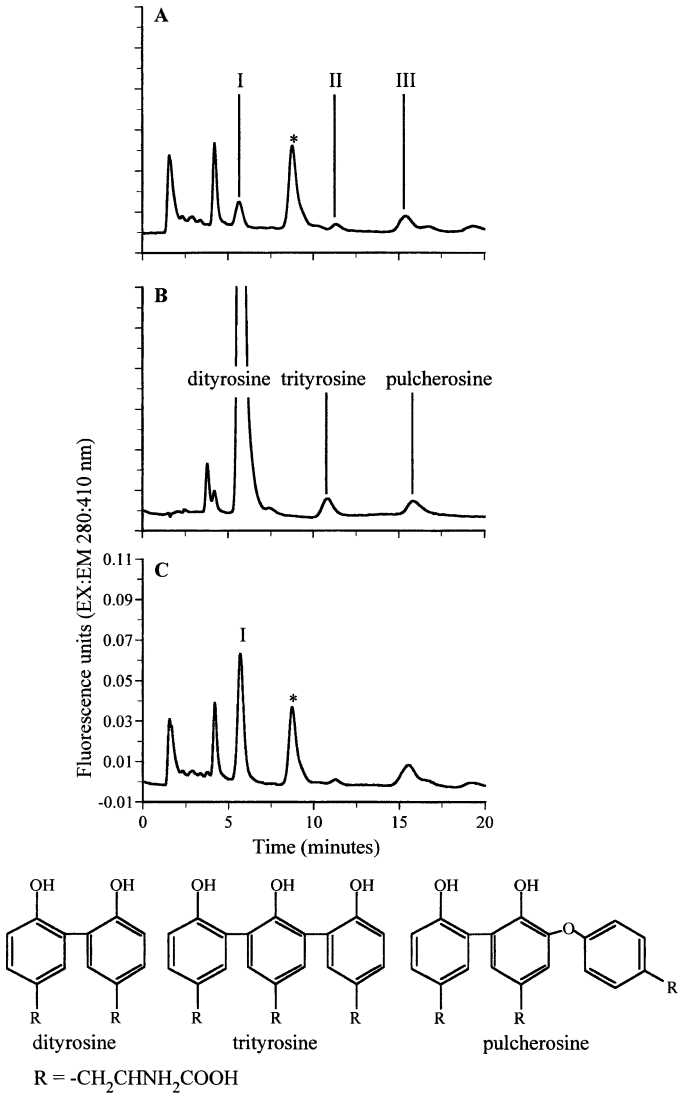

Fig. 6. Tyrosine oxidation catalyzed by (A) *Nereis virens* gut fluid. Oxidation products, peaks I–III, are identified as dityrosine, trityrosine, and pulcherosine on the basis of a (B) HRP-prepared standard, on the identification made by Jacob et al. (1996), and by (C) standard addition of dityrosine to (A). Peak marked with an asterisk is unidentified. See text for details.

incubated samples were identified by HPLC (Fig. 6A). HPLC/F of the dityrosine standard prepared with HRP gave three peaks with identical retention times (Fig. 6B), which are identified as dityrosine, trityrosine, and pulcherosine on the basis of the identification made by Jacob et al. (1996). Dityrosine in a gut fluid sample was identified by addition of a small aliquot of manually collected dityrosine from the HRP-prepared standard (Fig. 6C). A large peak at 8.7 min was not identified. It might be a second dimer, isodityrosine, of tyrosine oxidation, with the two tyrosine units linked by a biphenyl ether bridge (the two rightmost tyrosine units in pulcherosine on Fig. 6), which has been identified in plant cell wall glycoprotein (Fry 1982).

Antioxidant activity—Gut fluid from *N. virens* strongly reduced ethylene production from KMBA, even at a 1 : 1,000 dilution (Fig. 7). Ethylene concentration was significantly reduced with decreasing dilution of gut fluid and increasing glutathione concentration. At lower dilutions of gut fluid (1 : 50), a distinct induction period of 60 min was observed. Calculating the area under the curves for all dilutions of *N. virens* gut fluid and control experiments and using a gut fluid TAA concentration of 21.7 mg ml^{-1} gave a TOSC value of 1.82 ± 0.14 μg^{-1} TAA (n = 4).

Enzyme inhibition tests—Inactivation of a heme-containing enzyme can be brought about by the addition of a substance that can attach to the sixth coordination position of the metal atom in the heme group (e.g., NaCN). Inactivation of an oxidative enzyme can also result from addition of a reducing agent (e.g., $Na_2S_2O_5$), which reduces the active metal of the prosthetic group, or by addition of low–molecular weight nonenzymatic antioxidants (e.g., cysteine), which lower the amount of peroxide that can be reduced by the enzyme. NaCN, $Na_2S_2O_5$, and cysteine all inhibited the decolorization of RBBR by up to 35% (Fig. 8A–C). Although enzyme activity can also be reduced by heat inactivation, boiling gut fluid, but allowing it to cool to room temperature prior to use, had no effect on the RBBR decolorization (data not shown).

Discussion

The observed oxidative coupling potential of polychaete gut fluid and the tentative identification of a heme-containing

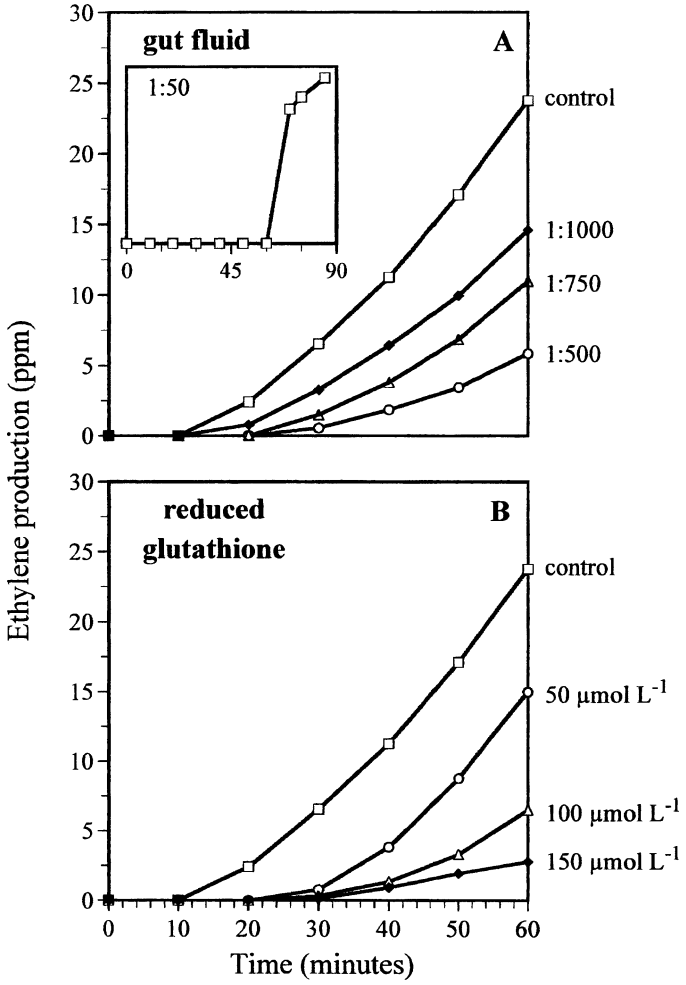

Fig. 7. Time course of ethylene production from oxidation of KMBA by peroxyl radicals in the presence of different dilutions of *Nereis virens* (A) gut fluid and (B) reduced glutathione.

oxidoreductive enzyme indicates that phenolic compounds, such as metabolites of PAHs, can undergo enzyme-catalyzed, oxidative coupling during gut passage in a marine deposit feeder. Binding of contaminants to humic matter will decrease the amount of material to interact with biota, both micro- and macrofauna, thus reducing toxicity of the compounds and rendering them resistant to further biotransformation (i.e., microbial degradation), and thus represents a new sink for organic contaminants in marine sediments. The putative enzyme was not isolated and purified but it is implied by removal of oxidative coupling potential after precipitation of the HMW protein fraction. An enzymatically catalyzed process is further corroborated by the high antioxidant capacity of unaltered gut fluid. Furthermore, identification of dityrosine as an oxidative coupling product (Fig. 6) suggests that gut passage could be important in creating humic-like polymers through the formation of stable covalent bonds, thereby forming recalcitrant organic matter in marine sediments. Because of its chemical stability and its widespread use as a marker for oxidative stress in human disease, dityrosine in marine sediments might prove a valuable marker for the influence of marine macrofauna on preservation of organic matter in the marine sediments.

Oxidative coupling—Oxidative coupling potential is here operationally defined as the decrease in recovery of pyrenol from aqueous solution after incubation with invertebrate gut fluid activated with H_2O_2. However, even without H_2O_2 activation, pyrenol recovery is <10% after 2 h of incubation with *N. virens* gut fluid (Fig. 4). Richnow et al. (1997) demonstrated efficient binding of four aromatic alcohols, including pyrenol, to soil humic substances with a HRP assay. Binding of other environmentally relevant chemicals (i.e., aliphatic and aromatic hydrocarbons, fatty acids, and aliphatic alcohols) to humic acid in a HRP/H_2O_2 assay was also tested, but only aromatic alcohols were found to be reactive (Richnow et al. 1997). The authors observed that cross-linking between naphthols (i.e., dimerization) was not significant in the presence of humic substances. Thus dimerization of pyrenol, at the micromolar concentration used in the assay presented here, seems unlikely with the high DOC commonly observed in gut fluid.

Of the four deposit-feeding marine invertebrates tested for oxidative coupling potential of pyrenol, only gut fluid from the sea cucumber, *P. californicus*, left significant amounts of dissolved pyrenol after the 1-h incubation period (Fig. 5). Echinoderms (*P. californicus*) and polychaetes (*A. marina*, *A. brasiliensis*, and *N. virens*) differ in terms of digestive

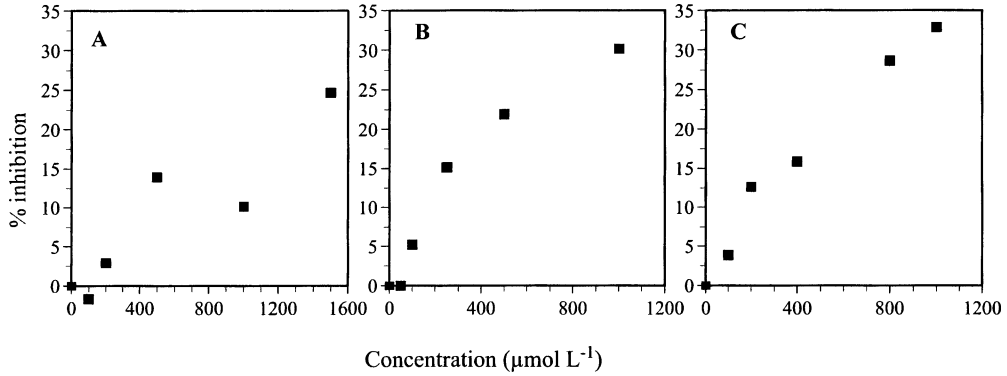

Fig. 8. Influence of various concentrations of enzyme inhibitors (A) NaCN, (B) $Na_2S_2O_5$, and (C) cysteine on the RBBR decolorization reaction (pH = 8).

strength (hydrolytic enzyme activity); digestive fluids from echinoderms tend to be weak, and those from polychaetes tend to be strong (Mayer et al. 1997). TAA content of gut fluid from the four species used in this experiment follow the same trend and can in part explain the variation in pyrenol recoveries in control treatments observed among them. Activities of deposit feeder digestive enzymes (i.e., protease, lipase, and esterase) generally correlate well with one another (Mayer et al. 1997), and if enzymes that cause oxidative coupling are similarly correlated, their activity would presumably follow a similar cross-phyletic pattern.

Pyrenol has been identified as the only hydroxylated metabolite of pyrene in species of deposit-feeding polychaetes (Giessing et al. 2003). Rapid intracellular turnover of pyrenol to carbohydrate and sulfate conjugates limits the amount of pyrenol leaking into the gut, thereby reducing its availability for coupling reactions. PAHs with more di- and trihydroxylated metabolites than pyrene (e.g., benzo[*a*]pyrene and fluoranthene) might undergo more significant oxidative coupling during gut passage.

Total oxyradical scavenging capacity of gut fluid and oxidoreductive enzymes—Loss of oxidative coupling potential after removal of HMW protein implies the presence of enzymes capable of inducing the observed reaction. A high TOSC value would be expected if the oxidative coupling reactions are a result of an antioxidant enzyme such as a peroxidase. *N. virens* gut fluid gave a TOSC value of 1.82 $\pm$ 0.14 per μg TAA. Previous studies have shown that HMW amino acids (i.e., proteins) constitute about half of TAA in *N. virens* gut fluid (Mayer et al. 1997), thus potentially doubling the TOSC value when normalized to HMW protein. Regoli and Winston (1998) reported TOSC values of 0.39 $\pm$ 0.02 μg^{-1} protein to 1.47 $\pm$ 0.08 μg^{-1} protein in rat liver and basal stalk of sea anemones (*Bunodosoma cavernata*), respectively, with the same method for estimation of TOSC. The same study showed TOSC values that were 60–90% higher in the digestive gland of mussel (*Mytilus edulis*) and pyloric ceca of starfish (*Leptasterias epichlora*) than in rat liver cytosolic fractions. Furthermore, the high TOSC value of gut fluid, and thus high antioxidant capacity, suggests that deposit feeder guts are places of significant ROS production, possibly resulting from ingestion of large amounts of sediment rich in redox-cycling transition metals and oxidation products of photolabile organic compounds.

Peroxidases are powerful antioxidants with a high oxidative coupling potential (Dunford 1999). At 1 : 50 dilution of gut fluid, a distinct induction period, in which ethylene formation from KMBA was totally inhibited or retarded relative to controls, was observed (*see* insert in Fig. 7, top panel) indicating the presence of fast-acting antioxidants (Winston et al. 1998). Other naturally occurring antioxidants (e.g., low molecular weight compounds such as vitamin C) might be present in the reaction mixture, competing with antioxidant enzymes for peroxyl radicals generated by ABAP. Humans, for example, secrete vitamin C into gastric juice (Halliwell and Gutterridge 1999).

Decolorization of RBBR has been extensively used to identify oxidoreductive enzyme activity, especially in fungi. The dependence of the gut fluid–catalyzed decolorization of RBBR on H_2O_2 and inhibition of the reaction with known inhibitors of oxidative enzymes (Fig. 8) corroborate the presence of enzymes. Furthermore, inhibition by cyanide suggests catalytic metal centers such as heme prosthetic groups. Enzyme activities are usually diminished by thermal breakdown of the enzyme molecule. Peroxidases are perhaps the most thermostable of all enzymes (Saunders et al. 1964), and the activity of heat-inactivated enzyme is often regained on cooling to room temperature (Lu and Whitaker 1974). This thermostability could explain our finding that heat inactivation, followed by cooling, had no effect on oxidative coupling activity. The combination of these results suggest that the enzyme responsible is a heme peroxidase.

It is also possible that dissolved metals or particulate metal oxides contribute significantly to oxidative coupling in gut fluid. Decolorization of RBBR can be catalyzed by metal ions (e.g., iron and copper) and H_2O_2 in Fenton-type reactions, and the effectiveness of a Fenton reaction is increased if the catalytic metal is chelated (Halliwell and Gutterridge 1999). As mentioned previously, deposit feeder gut fluids contain high concentrations of both metals and metal chelators (Chen et al. 2000), thus providing ideal conditions for Fenton-type reactions to occur. Fenton-type reactions produce highly reactive hydroxyl radicals ($OH^{\cdot}$), and the indiscriminate reactivity of $OH^{\cdot}$ would make both pyrene and pyrenol potential radical scavengers. If the proposed oxidative coupling reaction is driven by a Fenton-type reaction, pyrene would potentially be oxidized/lost from incubations with gut fluid activated with H_2O_2. We observed no loss of pyrene from gut fluid incubations activated with H_2O_2 compared with a DI nonactivated sample (Fig. 3). Thus, decolorization of RBBR is more likely a result of an enzymatic catalyzed oxidation.

Among metal oxides, hydroxides, and oxyhydroxides, manganese oxides are the most powerful catalysts in transformation of phenolic compounds to humic substances (Shindo and Huang 1982, 1984). Larson and Hufnal (1980) reported that dissolved metal cations, clays, and insoluble oxides promoted the polymerization of polyphenolic compounds and that stream water and sediment appear to catalyze the same reaction.

The purpose of the peroxidase or other enzymes with oxidative coupling potential in gut fluid of marine deposit-feeding invertebrates remains unclear. Although peroxidases occur in protozoa, Mollusca, Annelida, Echinodermata, and Vertebrata (Saunders et al. 1964; Dunford 1999), little is known about the presence or function of extrasomatic peroxidases in aquatic invertebrates. Defretin (1941) reported the presence of peroxidase in the mucus of the parapodial glands of nereids. On the basis of the distribution of the enzyme, Defretin (1941) argued that the observed activity was due to a peroxidase but did not isolate and identify the enzyme. Although Halliwell and Gutterridge (1990) concluded that extracellular antioxidant enzymes contribute little to the antioxidant status of human extracellular fluids, deposit feeder guts might represent an environment where extracellular, indeed extrasomatic, antioxidant enzymes are important factors in protecting against ROS produced as a consequence of the unique digestive environment.

Neuhauser and Hertenstein (1978) suggested that soil invertebrates use peroxidase enzymes as a defense mechanism against invasion of microbes. They also observed that peroxidase activity was present in most of the invertebrates (slugs, earthworms, millipedes) that feed on material rich in lignins, tannins, or both. Deposit-feeding macrofauna consume and digest sedimentary bacteria, and gut fluids from marine deposit-feeding polychaetes have bacteriolytic activities (Plante et al. 1996; Hymel and Plante 2000).

Peroxidase activity in gut fluid could also derive from of lysis of prey or the lining of the gut (i.e., digestive tract epithelial cells). Activities of cytosolic antioxidant enzymes can be up to 57 times higher in gut tissue than in somatic tissue of *A. marina* (Buchner et al. 1996). Deposit-feeding polychaetes are thought to retain gut fluid by antiperistaltic pumping (Mayer et al. 1997), with the potential to build up enzyme activities.

Enzyme-catalyzed oxidative coupling in animal guts not only suggests a mechanism for incorporation of PAHs into humic matter but also suggests a biological mechanism for the formation of aquatic humic material in general. Oxidative coupling of free tyrosine is not precluded, but dityrosine is readily formed by oxidative coupling of tyrosine residues in polypeptides and proteins (e.g., Michon et al. 1997; Audette et al. 2000). Formation of tyrosyl radicals can also initiate lipid peroxidation (Savenkova et al. 1994). Deposit feeders typically process large amounts of sediment (Cadée 1976; Lopez and Levinton 1987), exposing the top several centimeters of sediment to such a process on an annual basis.

Our findings imply that passage through digestive systems can influence system-level organic matter cycling. Most hypothesized pathways for the formation of refractory organic matter, such as melanoidin formation (Ishiwatari 1992) or lipid oxidation (Harvey et al. 1984), have focused on chemical conditions in ambient water and sediments. Reaction extents in these environments are limited by the low concentrations of both reactants and catalysts. The unique and intense chemical conditions found in digestive systems, however, set the stage for novel chemical reactions that could link organic precursors into condensates, perhaps with refractory character. For example, steryl chlorin esters (Talbot et al. 1999) might result from high esterase activities in guts that can synthesize as well as hydrolyze ester bonds. Oxidative coupling of aromatic alcohols, as demonstrated here, provides another example and suggests searching for other reactions in this intense reaction zone.

References

ABELE-OESCHAGER, D., R. OESCHAGER, AND H. THEEDE. 1994. Biochemical adaptations of *Nereis diversicolor* (Polychaeta) to temporarily increased hydrogen peroxide levels in intertidal sandflats. Mar. Ecol. Prog. Ser. **106:** 101–110.

ALLER, R. C., AND J. Y. ALLER. 1998. The effect of biogenic irrigation intensity and solute exchange on diagenetic reaction rates in marine sediments. J. Mar. Res. **56:** 905–936.

———, ———, AND P.F. KEMP. 2001. Effects of particle and solute transport on rates and extent of remineralization in bioturbated sediments, p. 315–333. *In* J. Aller, S. A. Woodin, and R. C. Aller [eds.], Organism–sediment interactions. Univ. of South Carolina Press.

ARFSTEN, D. P., D. J. SCHAEFFER, AND D. C. MULVENY. 1996. The effects of near ultraviolet radiation on the toxic effects of polycyclic aromatic hydrocarbons in animals and plants: A review. Ecotoxicol. Environ. Saf. **33:** 1–24.

AUDETTE, M., Y. BLOUQUIT, AND C. HOUEE-LEVIN. 2000. Oxidative dimerization of proteins: Role of tyrosine accessibility. Arch. Biochem. Biophys. **376:** 217–220.

BOLLAG, J.-M. 1992. Decontaminating soil with enzymes. Environ. Sci. Technol. **26:** 1876–1881.

———. 1983. Cross-coupling of humus constituents and xenobiotic substances, p. 127–142. *In* R. F. Christman and E. T. Gjessing [eds.], Aquatic and terrestrial humic materials. Ann Arbor Science Publishers.

———, J. DEC, AND P. M. HUANG. 1998. Formation mechanisms of complex organic structures in soil habitats. Adv. Agron. **63:** 237–266.

BUCHNER, T., D. ABELE-OESCHAGER, AND H. THEEDE. 1996. Aspects of antioxidant status in the polychaete *Arenicola marina*: Tissue and subcellular distribution, and reaction to environmental hydrogen peroxide and elevated temperatures. Mar. Ecol. Prog. Ser. **143:** 141–150.

CADÉE, G. C. 1976. Sediment reworking by *Arenicola marina* on tidal flats in the Dutch Wadden sea. Neth. J. Sea Res. **10:** 440–460.

CHEN, Z., L. M. MAYER, C. QUÉTEL, O. F. X. DONARD, R. F. L. SELF, P. A. JUMARS, AND D. P. WESTON. 2000. High concentrations of complexed metals in the guts of deposit feeders. Limnol. Oceanogr. **45:** 1358–1367.

DEFRETIN, R. 1941. Sur la présence de peroxidases dans le glandes a mucus parapodiales de *Nereis pelagicia* et de *Nereis irrorata.* C.R. Soc. Biol. **135:** 1258–1260.

DUNFORD, H. B. 1999. Heme peroxidases. Wiley-VCH.

FELTON, G. W. 1995. Oxidative stress of vertebrates and invertebrates, p. 356–434. *In* S. Ahmad [ed.], Oxidative stress and antioxidant defenses in biology. Chapman and Hall.

FILIP, Z., AND T. PREUSSE. 1985. Phenoloxidierende Enzyme—ihre Eigenschaften und Wirkungen im Boden. Pedobiologia **28:** 133–142.

FORBES, V. E., T. L. FORBES, AND M. HOLMER. 1996. Inducible metabolism of fluoranthene by the opportunistic polychaete *Capitella* sp. I. Mar. Ecol. Prog. Ser. **132:** 63–70.

FRY, S. C. 1982. Isodityrosine, a new cross-linking amino acid from plant cell-wall glycoprotein. Biochem. J. **204:** 449–455.

GIESSING, A., L. M. MAYER, AND T. L. FORBES. 2003. 1-Hydroxypyrene glucuronide as the major aqueous pyrene metabolite in tissue and gut fluid from the marine deposit-feeding polychaete *Nereis diversicolor.* Environ. Toxicol. Chem. **22:** 1107–1114.

GLENN, J. K., AND M. H. GOLD. 1983. Decolorization of several polymeric dyes by the lignin-degrading basidomycete *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. **45:** 1741–1747.

HALLIWELL, B., AND J. M. C. GUTTERRIDGE. 1990. The antioxidants of human extracellular fluids. Arch. Biochem. Biophys. **280:** 1–8.

———, AND ———. 1999. Free radicals in biology and medicine, 3rd. ed. Oxford Univ. Press.

HARVEY, G. R., D. A. BORAN, S. R. PIOTROWICZ, AND C. P. WEISEL. 1984. Synthesis of marine humic substances from unsaturated lipids. Nature **309:** 244–246.

HYMEL, S. N., AND C. J. PLANTE. 2000. Feeding and bacteriolytic responses of the deposit-feeder *Abarenicola pacifica* (Polychaeta: Arenicolidae) to changes in temperature and sediment food concentration. Mar. Biol. **136:** 1019–1027.

ISHIWATARI, R. 1992. Macromolecular material (humic substance) in the water column and sediments. Mar. Chem. **39:** 1–3.

JACOB, J. S., D. P. CISTOLA, F. F. HSU, S. MUZAFFAR, D. M. MUELLER, S. L. HAZEN, AND J. W. HEINECKE. 1996. Human phagocytes employ the myeloperoxidase–hydrogen peroxide system to synthesize dityrosine, trityrosine, pulcherosine, and isodityrosine by a tyrosyl radical-dependent pathway. J. Biol. Chem. **271:** 19,950–19,956.

JUMARS, P. A., AND R. A. WHEATCROFT. 1989. Responses of benthos to changing food quality and quantity, with a focus on deposit feeding and bioturbation, p. 235–253. *In* W. H. Berger, V. S. Smetacek, and G. Wefer [eds.], Productivity of the ocean: Present and past. Wiley.

LARSON, R. A. 1997. Naturally occurring antioxidants. Lewis.

———, AND J. M. HUFNAL. 1980. Oxidative polymerization of dissolved phenols by soluble and insoluble inorganic species. Limnol. Oceanogr. **25:** 505–512.

LEVINTON, J. 1995. Bioturbators as ecosystem engineers: Control of the sediment fabric, inter-individual interactions, and material fluxes, p. 29–51. *In* C. G. Jones and J. H. Lawton [eds.], Linking species and ecosystems. Chapman and Hall.

LIVINGSTONE, D. R., P. G. MARTINEZ, X. MICHEL, J. F. NARBONNE, S. OHARA, D. RIBERA, AND G. W. WINSTON. 1990. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis*-L, and other mollusks. Funct. Ecol. **4:** 415–424.

LOPEZ, G. R., AND J. S. LEVINTON. 1987. Ecology of deposit-feeding animals in marine sediments. Q. Rev. Biol. **62:** 235–253.

LU, A. T., AND J. R. WHITAKER. 1974. Some factors affecting rates of heat inactivation and reactivation of horseradish peroxidase. J. Food Sci. **39:** 1173–1178.

MALENCIK, D. A., J. F. SPROUSE, C. A. SWANSON, AND S. R. ANDERSON. 1996. Dityrosine: Preparation, isolation, and analysis. Anal. Biochem. **242:** 202–213.

MARCHAND, D., AND J. F. RONTANI. 2001. Characterisation of photo-oxidation and autoxidation products of phytoplanktonic monounsaturated fatty acids in marine particulate matter and recent sediments. Org. Geochem. **32:** 287–304.

MAYER, L. M., L. L. SCHICK, T. SAWYER, C. J. PLANTE, P. A. JUMARS, AND R. L. SELF. 1995. Bioavailable amino acids in sediments: A biomimetic, kinetics-based approach. Limnol. Oceanogr. **40:** 511–520.

———, AND OTHERS. 1996. Bioavailability of sedimentary contaminants subject to deposit-feeder digestion. Environ. Sci. Technol. **30:** 2641–2645.

———, L. L. SCHICK, R. F. L. SELF, P. A. JUMARS, R. H. FINDLAY, Z. CHEN, AND S. SAMSON. 1997. Digestive environments of benthic macroinvertebrate guts: Enzymes, surfactants and dissolved organic matter. J. Mar. Res. **55:** 785–812.

———, P. JUMARS, M. J. BOCK, Y.-A. VETTER, AND J. L. SCHMIDT. 2001*a*. Two roads to Sparagmos: Extracellular digestion of sedimentary food by bacterial inoculation versus deposit-feeding, p. 335–348. *In* J. Aller, S. A. Woodin, and R. C. Aller [eds.], Organism–sediment interactions. Univ. of South Carolina Press.

———, D. P. WESTON, AND M. J. BOCK. 2001*b*. Benzo-*a*-pyrene and zinc solubilization by digestive fluids of benthic invertebrates—a cross phyletic study. Environ. Toxicol. Chem. **20:** 1890–1900.

MCKNIGHT, D. M., AND G. R. AIKEN. 1998. Sources and age of aquatic humus, p. 9–39. *In* D. O. Hessen and L. J. Tranvik [eds.], Aquatic humic substances. Ecology and biogeochemistry. Springer.

MEYERHOFFER, S. M., AND L. B. MCGOWN. 1991. Microenvironments of fluorescence probes in sodium taurocholate and taurodeoxycholate bile salt media. Anal. Chem. **63:** 2082–2086.

MICHON, T., M. CHENU, N. KELLERSHON, M. DESMADRIL, AND J. GUEGUEN. 1997. Horseradish peroxidase oxidation of tyrosine-containing peptides and their subsequent polymerization: A kinetic study. Biochemistry **36:** 8504–8513.

NAIDJA, A., P. M. HUANG, AND J.-M. BOLLAG. 2000. Enzyme–clay interactions and their impact on transformations of natural and anthropogenic organic compounds in soil. J. Environ. Qual. **29:** 677–691.

NEUHAUSER, E. F., AND R. HERTENSTEIN. 1978. Reactivity of macroinvertebrate peroxidase with lignins and lignin model compounds. Soil Biol. Biochem. **10:** 341–342.

PASTI, M. B., AND D. L. CRAWFORD. 1991. Relationships between the abilities of streptomycetes to decolorize three anthron-type dyes and degrade lignocellulose. Can. J. Microbiol. **37:** 902–907.

PEARSON, T. H., AND R. ROSENBERG. 1978. Macrobenthic succession in relation to organic enrichment and pollution of the marine environment. Oceanogr. Mar. Biol. Ann. Rev. **16:** 229–311.

PENNATHUR, S., V. JACKSON-LEWIS, S. PRZEDBORSKI, AND J. W. HEINECKE. 1999. Mass spectrometric quantification of 3-nitrotyrosine, ortho-tyrosine, and o,o′-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin-treated mice, a model of oxidative stress in Parkinson's disease. J. Biol. Chem. **274:** 34,621–34,628.

PLANTE, C. J., L. M. MAYER, AND G. M. KING. 1996. The kinetics of bacteriolysis in the gut of the deposit feeder *Arenicola marina.* App. Environ. Microbiol. **62:** 1051–1057.

REGOLI, F., AND G. W. WINSTON. 1998. Applications of a new method for measuring the total oxyradical scavenging capacity in marine invertebrates. Mar. Environ. Res. **46:** 439–442.

RICHNOW, H. H., R. SEIFERT, J. HEFTER, M. KASTNER, B. MAHRO, AND W. MICHAELIS. 1994. Metabolites of xenobiotica and mineral oil constituents linked to macremolecular organic matter in polluted environments. Org. Geochem. **22:** 671–681.

———, ———, M. KASTNER, B. MAHRO, B. HORSFIELD, U. TIEDGEN, S. BOHM, AND W. MICHAELIS. 1995. Rapid screening of PAH-residues in bioremediated soils. Chemosphere **31:** 3991–3999.

———, ———, J. HEFTER, M. LINK, W. FRANCKE, G. SCHAEFER, AND W. MICHAELIS. 1997. Organic pollutants associated with macromolecular soil organic matter: Mode of binding. Org. Geochem. **26:** 745–758.

RONTANI, J. F., AND D. MARCHAND. 2000. Photoproducts of phytoplanktonic sterols: A potential source of hydroperoxides in marine sediments? Org. Geochem. **31:** 169–180.

SAUNDERS, B. C., A. G. HOLMES-SIEDLE, AND B. P. STARK. 1964. Peroxidase. The properties and uses of a versatile enzyme and some related catalysts. Butterworths.

SAVENKOVA, M. I., D. M. MUELLER, AND J. W. HEINECKE. 1994. Tyrosyl radical generated by myeloperoxidase is a physiological catalyst for the initiation of lipid-peroxidation in low-density-lipoprotein. J. Biol. Chem. **269:** 20,394–20,400.

SHINDO, H., AND P. M. HUANG. 1982. The role of Mn(IV) oxide in abiotic formation of humic substances in the environment. Nature **298:** 363–365.

———, AND ———. 1984. Catalytic effect of manganese(IV), iron(III), aluminum, and silicon oxides on the formation of phenolic polymers. Soil Sci. Soc. Am. J. **48:** 927–934.

TALBOT, H. M., R. N. HEAD, R. P. HARRIS, AND J. R. MAXWELL. 1999. Distribution and stability of steryl chlorin esters in copepod feacal pellets from diatom grazing. Org. Geochem. **30:** 1163–1174.

VYAS, B. R. M., AND H. P. MOLITORIS. 1995. Involvement of an extracellular H_2O_2-dependent ligninolytic activity of the white root fungus *Pleurotus ostreatus* in the decolorization of Remazol Brilliant Blue R. Appl. Environ. Microbiol. **61:** 3919–3927.

WEBER, W. J., JR., AND Q. HUANG. 2003. Inclusion of persistent organic pollutants in humification processes: Direct chemical incorporation of phenanthrene via oxidative coupling. Environ. Sci. Technol. **37:** 4221–4227.

WINSTON, G. W., AND R. T. DIGIULIO. 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol. **19:** 137–161.

———, F. REGOLI, A. J. DUGAS, J. H. FONG, AND K. A. BLANCHARD. 1998. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. Free Radical Bio. Med. **24:** 480–493.

Received: 25 June 2003
Accepted: 12 January 2004
Amended: 23 January 2004