Vol. 163: 179–191, 1998 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published March 12

Temporal fluctuations and spatial gradients of environmental P_{O_2}, temperature, H_2O_2 and H_2S in its intertidal habitat trigger enzymatic antioxidant protection in the capitellid worm *Heteromastus filiformis*

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ABSTRACT: Activity levels of 2 antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD), and the rate of oxygen consumption were investigated in body wall tissue of the capitellid polychaete *Heteromastus filiformis* in response to the variability of abiotic factors in the worm's intertidal habitat. A head-down deposit feeder, *H. filiformis* displayed oxyconformity between 1.3 and 13 kPa (10 to 100 torr) P_{O_2} under laboratory conditions. An extremely low standard metabolic rate (SMR) equivalent to an oxygen consumption of 0.1 µmol O_2 g^{-1} fw h^{-1} (fw = fresh weight) is consistent with the capacity of the worms to colonize anoxic and potentially sulphidic sedimentary environments. Maximal $\dot{M}_{O_2}$ was 0.3 ± 0.09 µmol g^{-1} fw h^{-1} for small (<300 mg body fw) and 0.25 ± 0.09 µmol g^{-1} fw h^{-1} for large (>500 mg body fw) worms. CAT and SOD activities were higher in summer than in winter worms. Under laboratory conditions, SOD activity in winter worms was not inducible upon acclimation to elevated temperatures, while CAT activity was significantly higher at 20 than at 5°C. Summer worms were unaffected by temperature reduction with respect to CAT activities, while SOD activity was significantly reduced upon cooling. Under laboratory conditions, hypoxia as well as elevated P_{O_2} led to a significant increase in CAT activity, while changes in SOD activity were marginal. Experimental hydrogen peroxide (H_2O_2) exposure resulted in an increase in CAT activity, whereas hydrogen sulphide (H_2S) led to a decrease in CAT activity only if applied under anoxia. SOD activities of *H. filiformis* were insensitive to H_2S *in vivo*. *In situ* exposure to elevated H_2O_2 concentrations confirmed that just 1 tidal emersion period was sufficient to cause the inducing effect of reactive oxygen species on CAT activities in the worms' natural habitat. It is concluded that short-term changes in CAT activity are triggered by specific environmental stress factors, like fluctuating P_{O_2} and hydrogen peroxide concentrations in the upper sediment layer. In contrast, SOD did not respond spontaneously under experimental conditions, but under *in situ* conditions at the sediment surface of an intertidal sandflat a shift of SOD activity occurred towards the end of an ebb tide emersion period, which led to an increase of SOD activity in the tail compared to the head end of the worms. It is hypothesized that short-term variations in P_{O_2} and temperature at the sediment surface, in combination with the vertical gradients of P_{O_2}, H_2S, temperature, and pH, elicit the observed changes in SOD activity.

KEY WORDS: *Heteromastus filiformis* · Antioxidant enzymes · P_{O_2} · Temperature · Hydrogen peroxide · Hydrogen sulphide

INTRODUCTION

Oxidative stress is not confined to organisms in well-oxygenated or hyperoxic environments. Invertebrates colonizing marine sediments with very low oxygen concentrations in the pore water are obviously in need of the same system of antioxidant protection as organisms living under fully oxidized conditions (Morrill et al. 1988, Buchner et al. 1996, Viarengo et al. 1998). Oxidative stress under hypoxic conditions can be a consequence of the prevailing hypoxia itself, as well as of ex-

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treme short-term variability of oxygen concentrations in the water. Photosynthetic activity of superficial microalgal mats during daytime can produce oxygen supersaturation in intertidal pools and at the sediment surface in places where suboxic or even anoxic conditions may prevail during the night (de Wit et al. 1989, Fenchel & Finlay 1995). Animals living in the sediment underneath such tidal pools have, under steady state conditions and with sufficient oxygen supply, a strictly aerobic metabolism, although colonizing low oxygen environments. Although some bivalves and polychaetes can tolerate several weeks of hypoxia and even anoxia on an anaerobic metabolism (Theede 1973, Brinkhoff et al. 1983), they try to keep the microenvironment inside their burrows oxidized by irrigation with surface water, or stay right beneath the sediment surface to keep their respiratory tissues in direct contact with the overlying water.

It has been shown that hypoxia increases oxidative stress in marine invertebrates basically via the same biochemical processes described for vertebrates (Jones 1985). These include autoxidation of haemoglobin (Hb) (Misra & Fridovich 1972, Caughey & Watkins 1985, Abele-Oeschger & Oeschger 1995) and also of partly reduced co-substrates of the mitochondrial respiratory chain (Rifkind et al. 1993). Moreover, fluctuating P_{O_2} in intertidal areas may have an effect comparable to ischaemia-reperfusion injury in vertebrates, in triggering superoxide radical liberation via enzymatic and non-enzymatic reactions (Halliwell & Gutteridge 1985, Storey 1996).

The present study deals with oxygen radical stress and antioxidant defense in the marine infaunal polychaete *Heteromastus filiformis*, which colonizes intertidal sediments at high population densities (up to 7000 ind. m^{-2}; Hartmann-Schröder 1971). The deposit feeder *H. filiformis* constructs permanent sedimentary tubes with a vertical orientation, reaching down to approximately 30 cm below the sediment surface (Cadée 1979). A head-down feeder, *H. filiformis* uses refractory organic material buried in deeper anoxic sediment layers as the only carbon source (Clough & Lopez 1993). In contrast to most other tube-dwelling polychaetes, *H. filiformis* does not oxidize its burrow wall through irrigation, but takes up oxygen through the gill-like parapods at its rear end, which the worms extend periodically into the oxidized sediment water boundary layer (Hartmann-Schröder 1971, Clough et al. 1993). If microalgae grow at the sediment surface, P_{O_2} and pH will change during tidal emersion due to photosynthetic activity (Gnaiger et al. 1978, de Wit et al. 1989). Oxygen supersaturation can be as high as 300% (Fenchel & Finlay 1995) while pH in the photosynthetic surface microlayers will increase to more alkaline values, occasionally exceeding pH 9.0 (Gnaiger et al. 1978).

The polychaete *Heteromastus filiformis* possesses cellular Hb of high oxygen affinity and a P_{50} as low as 0.68 torr (0.09 kPa) has been determined in native coelomic fluid, as well as in haemolysates (Pals & Pauptit 1979), reflecting adaptation of the worm to very low environmental oxygen concentrations. *H. filiformis* has been shown to endure low oxygen conditions for a few days (Clough & Lopez 1993) and, moreover, to keep its metabolism aerobic even in the presence of sulphide as long as oxygen is available at the sediment surface (Oeschger & Vismann 1994). Oeschger & Vismann (1994) found that *H. filiformis* can partly cover its energy demands via mitochondrial sulphide oxidation, which is coupled to ATP production. This can only be achieved by exploiting a vertical P_{O_2}, pH and H_2S gradient between the sediment surface and the deeper anoxic sediment strata. Pals & Pauptit (1979) found that pH in sediment pore water near *H. filiformis* burrows decreases from pH 7.94 at 10 cm to 7.70 at 30 cm sediment depth, whereas oxygen penetrates only the upper few millimeters of surface sediment (Fenchel & Finlay 1995). The external gradient obviously tunes an internal pH gradient in the coelomic fluid of *H. filiformis* with a lower pH between 6.3 and 6.6 in the head and between 6.7 and 7.0 in the tail end (Pals & Pauptit 1979). The idea is that the lower pH in the head region together with a moderate Bohr effect [Bohr factor (ϕ) = $-0.34\Delta\log P_{50}/\Delta pH$], found for *H. filiformis* Hb by Pals & Pauptit (1979), facilitates oxygen delivery to the tissues in deeper anoxic sediment layers.

The objective of the present study was not only to elucidate the oxidative stress arising from the exposure of a species to time dependent fluctuations of environmental P_{O_2} and other physical parameters in intertidal surface sediments, but also to assess how a spatial P_{O_2} and pH gradient might affect reactive oxygen species (ROS) production in body tissues. In order to clarify whether *Heteromastus filiformis* reacts as an 'oxyconformer' or an 'oxyregulator', we measured oxygen consumption of individuals at variable P_{O_2} levels.

The polychaete *Heteromastus filiformis* provides an ideal model organism to study the effects of the abiotic microenvironment on animal pysiology, as it does not strive to actively 'homogenize' its physical environment through bioirrigation, but makes use of the gradient between its upper oxidized tail end and its head located in anoxic, sulphide-containing deeper sediment strata. Apparently, this modus vivendi increases the worm's risk of suffering oxidative stress. In a comparative study on SOD (superoxide dismutase) activity in benthic invertebrates, *H. filiformis* had the highest activities of this oxygen radical quencher in whole animal extracts (Abele-Oeschger 1996).

MATERIAL AND METHODS

Specimens. *Heteromastus filiformis* specimens for laboratory experiments were collected on an intertidal sandflat on the North Sea Wadden Coast (Dorum-Neufeld), north of Bremerhaven, Germany. The polychaetes are very thin (∅ 1 mm) and up to 10 cm long, so that sampling of intact individuals could only be achieved by carefully mixing sediment with seawater and retrieving specimens by hand or with tweezers from the slurry.

Description of sampling locations. Most *in situ* sampling was done on an intertidal sandflat. In one experiment, worms from the sandflat location were compared to worms collected from a mudflat site with high organic loading. The sandflat is situated about 800 m from the coast, where tidal currents are unaltered. Pore water content was 20% of sediment wet weight. The fine grained silt material (<63 µm) constituted less than 7% of the sediment mineral fraction and the organic content was around 1% of sediment dry weight throughout the upper 25 cm of this sandflat. H_2S pore water concentrations never exceeded 150 µmol l^{-1}.

The mudflat is located 300 m from the coast and is situated next to a stone dam (groyne) which protrudes some 400 m into the Wadden Sea, to slow down tidal water movements. Pore water content was 30% of sediment wet weight. Reduced current velocity leads to a local enrichment of fine grained material (particle size <63 µm), which amounts to 40–50% of the sediment mineral fraction. The organic content was between 4 and 5% of sediment dry weight in the sediment surface and 2% in 10 cm sediment depth. High organic loading increases sediment oxygen demand and may support a local increase of hydrogen sulphide concentrations in sediment pore water (between 50 and 450 µmol l^{-1}).

Experimental setup for laboratory investigations. ***Determination of oxygen uptake at different P_{O_2} levels:*** $\dot{M}_{O_2}$ (oxygen consumption rate) of individual specimens of 280 to 660 mg body fw (fresh weight), collected in August 1996, was measured in closed respiration chambers with an adjustable volume of between 1.5 and 2 ml each. The chambers were thermostatted to 10°C and oxygen consumption recorded with polarographic oxygen electrodes (Eschweiler, Germany) connected to an oxymeter (M200, Eschweiler, Germany) and a Linseis chart recorder. Seawater used in the experiments was filtered through 0.2 µm cellulose-acetate filters to minimize bacterial respiration. P_{O_2} was adjusted by aerating the incubation water. Worms were allowed to respire in these chambers and $\dot{M}_{O_2}$ was calculated for discrete increments of the P_{O_2} curve.

Temperature incubations: Winter worms for temperature incubations were collected in January 1995 at an *in situ* sediment temperature of 2°C. These worms were exposed to elevated temperatures (10 and 20°C) for 14 d. Control worms were kept at 5° C for 14 d (n = 6 to 9). Summer worms were collected in August 1995 at a sediment temperature between 20 and 25°C and 2 groups were exposed to lower temperatures (10 and 0°C) over 14 d in the laboratory. Control worms were kept at 20°C for 14 d (n = 8 or 9). All *T* (temperature) incubations were carried out in aquaria with sieved sediment from the sampling area and aerated seawater (S: 25‰, normoxia).

Experimental hydrogen peroxide exposure: Experimental exposure to elevated hydrogen peroxide concentrations was carried out at 10°C in filtered seawater without sediment. A first experiment, in which worms were exposed to 6 µmol l^{-1} hydrogen peroxide over a period of 6 h was conducted in March 1995. A second experiment was carried out in June 1995 when worms collected from the natural environment already had higher basal catalase activity. Consequently, these worms were exposed to higher H_2O_2 concentrations (10 µmol l^{-1}) over a total period of 12 h. Both concentrations applied are about twice as high as the maximum natural H_2O_2 concentrations we measured on the intertidal mudflats during the respective seasons.

Experimental hydrogen sulphide exposure: Sulphidic incubations were carried out in the laboratory at 12°C with 100 µmol l^{-1} hydrogen sulphide under oxic and under anoxic conditions over 6 h. Seawater used in these experiments was buffered with HEPES (Biomol) and the pH was adjusted to 8.1 with 1 N HCl after addition of sulphide. Sulphide was added from a 10 mmol l^{-1} stock solution of Na_2S in distilled water that had previously been equilibrated with nitrogen. This stock solution was kept in air-tight bottles and the concentration was controlled using the spectrophotometric method of Cline (1969).

Anoxic incubations were carried out in air-tight glass bottles. H_2S concentrations were adjusted after 3 h. Oxic incubations were run in a flow-through system. Sulphide stock solution was mixed with oxygenated seawater just before entering the flow-through incubation chamber (350 ml), using a peristaltic pump (Ismatec, Germany).

Worms were kept in individual compartments inside the incubation chamber. Hydrogen sulphide concentrations were measured after leaving the incubation chamber and readjusted to 100 µmol l^{-1} if necessary. Oxic and anoxic controls were run without addition of sulphide under the respective experimental conditions.

Analyses. ***Analysis of H_2O_2 and H_2S in surface water:*** Hydrogen peroxide was analyzed fluorimetri-

cally in water samples of surface pools, using scopoletin (7-hydroxy-6-methoxy-2H-benzopyran) as a fluorescence indicator (Andreae 1955) in a peroxidase catalyzed reaction (Kieber & Helz 1986, Cooper et al. 1989). Analyses were completed within 1 h after sampling. The method is described in detail in Abele-Oeschger et al. (1997).

H_2S was analyzed photometrically in pore water samples according to the method of Cline (1969). Pore water was sampled with a stainless steel pore water sampler from 10, 20 and 30 cm sediment depth.

Enzymatic assays: Catalase (CAT, E.C. 1.11.1.6.) activity was analyzed in whole worm extracts comprising body wall and coelomic fluid after Aebi (1985). Samples were ground in liquid nitrogen and homogenized in 50 mmol l^{-1} potassium phosphate buffer (pH 7.0, 1:10 tissue/buffer) including 1 part of 1% Triton-X100 solution to 9 parts of buffer. The catalase assay was conducted at 25°C using potassium phosphate buffer (50 mmol l^{-1}, pH 7.0) and H_2O_2 as substrate at 240 nm. A catalase standard was purchased from Boehringer, Mannheim (2600 U mg^{-1}). One unit of catalase is defined as the amount of enzyme that decomposes 1 µmol H_2O_2 min^{-1} under the assay conditions. Measurements of isolated coelomic fluid revealed that the haemolymph of *Heteromastus filiformis* is void of catalase activity, so that the activity measured in whole body extracts was actually confined to the body wall compartment of the polychaete.

Superoxide dismutase (SOD, E.C. 1.15.1.) activity was determined according to Marklund & Marklund (1974) in freshly collected tissues. Freeze storage, also in liquid nitrogen, was found to reduce SOD activity in the samples. SOD was analyzed in whole worm extracts as well as in isolated coelomic fluid. These were obtained from individual worms using 5 µl micropipettes. For measurements of whole tissue extracts, worms were cut into pieces and homogenized in 0.05 M Tris-succinate buffer, pH 8.2 at a 1:3 tissue/buffer ratio. Coelomic fluid was analyzed directly after retrieval without further processing. A detailed description of the assay has been given in Abele-Oeschger (1996). A SOD standard was purchased from Fluka, Germany. One unit of SOD reduces the autoxidation rate of an 8 mM pyrogallol solution by 50% at pH 8.2 and 25°C.

Tissue protein content: Enzyme activities are predominantly given in U mg^{-1} fw. Only for the seasonal comparison were data related to both tissue fresh weight and tissue protein content. This was done to facilitate comparison with other studies. Protein content was measured according to Bradford (1976).

Determination of Hb content: Hb was analyzed in whole tissue extracts. Fresh tissues were homogenized in Ringer solution (1:10, tissue/buffer) and centrifuged for 5 min at 10000 × *g*. Hb content was determined spectrophotometrically in the supernatant at 540 nm, using the molar extinction coefficient given by Dangott & Terwilliger (1986) for the Hb of the polychaete *Euzonus mucronata* ($\varepsilon_{mmol\,l^{-1}} = 14$). The concentration was then recalculated to Hb content per g tissue fresh weight of the whole worm extract.

RESULTS

Oxygen consumption at different P_{O_2}

Fig. 1 shows $\dot{M}_{O_2}$ as a function of P_{O_2} for (a) small and (b) large *Heteromastus filiformis* between 0 and 19.9 kPa (150 torr). The trend within the data points confirms what is expected for an oxyconformer according to Pörtner & Grieshaber (1993), i.e. a linear $\dot{M}_{O_2}/P_{O_2}$ relationship between a lower (≈ 1.3 kPa or 10 torr) and an upper critical P_{O_2} ($P_c \approx 10.6$ kPa or 80 torr). The low $\dot{M}_{O_2}$ of 0.1 ± 0.04 µmol g^{-1} fw h^{-1} at 1.3 kPa (10 torr) in both size groups characterizes the standard metabolic rate, i.e. the lowest rate of a completely aerobic metabolism (P_c between 1.2 and 3 kPa, where at some point the worms will switch to anaerobiosis). The upper critical P_{O_2}, at which $\dot{M}_{O_2}$ becomes independent of the environmental oxygen concentration, defines a saturation level of the oxyconformer's respiratory mechanisms with oxygen under resting conditions far above the lower critical P_{O_2}. Above the upper P_c, the $\dot{M}_{O_2}/P_{O_2}$ relationship steadied at 0.3 µmol g^{-1} fw, which represents maximal oxygen uptake of small individuals at high P_{O_2}. Large animals with a body weight of more than 500 mg displayed a $\dot{M}_{O_2}/P_{O_2}$ pattern similar to that of smaller specimens. Maximal $\dot{M}_{O_2}$ values of large worms were somewhat lower than those of small worms, amounting to 0.25 µmol g^{-1} fw h^{-1} above 13.3 kPa (100 torr).

Effects of variable abiotic factors on antioxidant enzymes under experimental conditions

The effects of temperature, hydrogen peroxide exposure and P_{O_2} on maximal activities of the enzymatic antioxidants SOD and CAT were tested under controlled laboratory conditions. An important difference to the natural environment was that the experimental conditions were not designed to imitate the vertical gradients of abiotic parameters that are found in the surface layers of the sediment.

Antioxidant enzyme (AOE) activities were generally assayed at 25°C, in order to demonstrate acclimation of the antioxidant potential of different experimental groups of worms by comparison at a standard temper-

ature. Thus, our data do not represent true rates, measured at *in situ* temperature, but rather normalized activity levels.

Temperature

Cold acclimated winter worms showed no uniform reaction when exposed to elevated temperatures (Fig. 2a). CAT remained more or less unchanged when temperature was increased from 5 to 10°C over 14 d, while at an incubation temperature of 20°C over the same period, the worms had significantly higher CAT activities ($p < 0.01$). In contrast, warm acclimated summer worms collected from the habitat at 20°C (summer controls; Fig. 2b) displayed slightly lower maximal CAT activities than after cooling to 0°C over 14 d.

Winter SOD activities were generally lower than summer activities and, moreover, completely independent of short-term temperature changes. However, in summer specimens low temperatures had a negative effect on SOD activity. A significant decrease of maximal SOD activities occurred upon cooling from 20 to 10°C ($p < 0.05$) and to 0°C ($p < 0.01$) over 14 d.

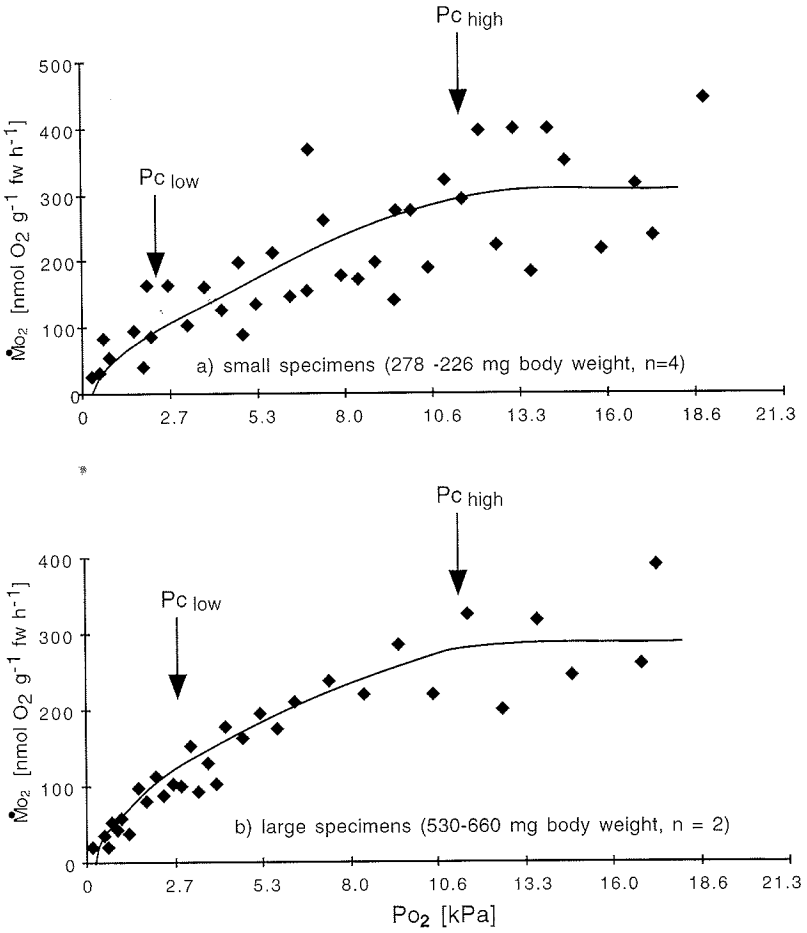

Fig. 1. *Heteromastus filiformis*. Oxygen consumption rates ($\dot{M}_{O_2}$) versus environmental P_{O_2} in (a) small (278 to 336 mg body weight, n = 4) and (b) large (530 to 660 mg body weight, n = 2) worms. Data from August and September 1996

P_{O_2}

To test the effect of different P_{O_2} levels on AOE activities, winter worms (February 1995, pre-adapted to 10°C over 7 d) were incubated for 6 h and for 24 h at 15°C in a flow-through chamber without sediment. P_{O_2} was adjusted to 0, 1.3, 6.7 and 21 kPa (= 0, 10, 50 and 158 torr) using a gas mixing system (Wösthoff, Germany). Nitrogen, oxygen and carbon dioxide were mixed so that P_{O_2} could be adjusted, while P_{CO_2} was kept constant (at 0.31 kPa).

The P_{O_2} in the very small sedimentary tubes of *Heteromastus filiformis* has never been quantified *in situ*. Pals & Pauptit (1979) measured the P_{O_2} in the interstitial water next to *Heteromastus filiformis* burrows and found it to be below 1 torr in 10 cm sediment depth and 0 torr in 30 cm sediment depth. Neira & Höpner (1994), recording sediment redox potential in a natural sandflat colonized by *H. filiformis*, found that the oxygenated horizon ($E_h > 300$ mV) in winter (February) reaches down to 3 cm sediment depth. During summer, except for the very thin surface layer, the redox potential of the sediment surface (0 to 6 cm sediment depth) was even lower ($E_h = 100$ to 300 mV), which means that oxygen was essentially absent throughout the burrow (Fenchel & Finlay 1995). Thus a P_{O_2} of between 1.3 and 6.7 kPa (10 and 50 torr) was used as control in the experiments, while 0 kPa represents anoxia and 21 kPa (158 torr) may already represent hyperoxic conditions for this species.

Fig. 3 shows the effect of different P_{O_2} values on *Heteromastus filiformis* SOD and on CAT activities during the 2 incubation experiments. The effects of low and high P_{O_2} on SOD activity were only marginal (Fig. 3b). The 6 h exposure to 50 and 158 torr resulted in a slight increase of SOD activity compared to control levels (10 torr) and to hypoxia. The effects were far more pronounced when CAT activity was measured in worms kept at high and low P_{O_2} (Fig. 3a). Lowest enzyme

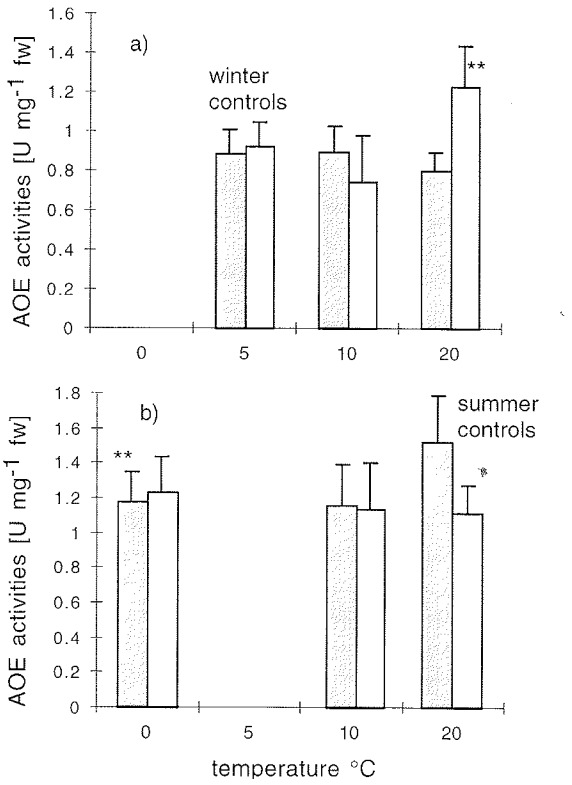

Fig. 2. *Heteromastus filiformis*. AOE activities (shaded bars: superoxide dismutase, SOD; open bars: catalase, CAT) in whole body extracts versus experimental temperature in summer and winter specimens. Winter worms were collected in January 1995 at an *in situ* sediment temperature of 2°C. These worms were exposed to elevated temperatures (10 and 20°C) for 14 d. Control worms were kept at 5°C for 14 d, (n = 6 to 9). Summer worms were collected in August 1995 at a sediment temperature between 20 and 25°C and exposed to lower temperatures (10 and 0°C) over 14 d in the laboratory. Control worms were kept at 20°C for 14 d, (n = 8 or 9). **Significant difference to control group ($p < 0.01$)

activities were measured at control levels (10 torr) and at 50 torr, whereas a significant increase of CAT activity was found during long- and short-term incubation under anoxia and also hyperoxia (158 torr).

Table 1. *Heteromastus filiformis*. Superoxide dismutase (SOD) and catalase (CAT) activity in U mg^{-1} fw (fresh weight) after oxic (158 torr) and anoxic (0 torr) incubation with hydrogen sulphide (100 µmol l^{-1}, 6 h, 10°C, n = 6). *Significant reduction ($p < 0.05$) of the enzymatic activity compared to the normoxic control group with or without sulphide

	H_2S anoxic	Anoxic controls	H_2S oxic	Oxic controls
SOD	1.21 ± 0.14*	1.23 ± 0.14*	1.40 ± 0.25	1.50 ± 0.17
CAT	1.21 ± 0.11*	1.48 ± 0.33	1.51 ± 0.26	1.74 ± 0.36

Hydrogen peroxide

Both incubations, carried out during spring (6 µmol l^{-1}, 6 h) and summer (10 µmol l^{-1}, 12 h), resulted in a significant increase of CAT activity above control levels (Fig. 4). The absolute increase was even more pronounced in the worms collected in June, although the percent increase was approximately the same (70 to 80%) during both incubations.

Hydrogen sulphide

Table 1 shows the results of the normoxic and the anoxic hydrogen sulphide incubations on the activities of the antioxidant enzymes SOD and CAT. H_2S had no significant effect on enzyme activities as long as oxygen was present. Anoxia with or without H_2S caused a significant decrease of SOD activity when compared to oxic controls. CAT remained unaffected by H_2S under normoxia and was only reduced by a combination of H_2S and anoxic exposure. The data corresponded to what was previously found in the laboratory experiment under different P_{O_2} levels, in which anoxia and normoxia of 21 kPa (158 torr, which may equal hyperoxia for *Heteromastus filiformis*) both resulted in elevated CAT activities (Fig. 3).

Impact of variable abiotic factors on AOE activities *in situ*

Summer versus winter conditions

Many abiotic factors vary between summer and winter in intertidal areas, the most decisive of which is the difference in sediment temperature. In January 1995, pore water temperatures (10 to 15 cm sediment depth) of 2°C prevailed throughout the day. In late spring, temperatures had increased to between 10 and 17°C,

Table 2. *Heteromastus filiformis*. Seasonal comparison of antioxidant enzyme activities (in U mg^{-1} fw; values in parantheses are U mg^{-1} protein) in whole worms. Data from winter and summer specimens collected in 1995. **Significant difference ($p < 0.01$) to winter worms (n = 7)

	SOD	CAT
January	0.41 ± 0.22 (16.97 ± 5.75)	0.66 ± 0.25 (20.74 ± 6.06)
May	2.17 ± 0.61** (62.62 ± 14.53)	1.19 ± 0.26** (47.03 ± 19.97)
August	2.07 ± 0.16** (66.65 ± 15.29)	2.11 ± 0.38** (43.93 ± 7.04)

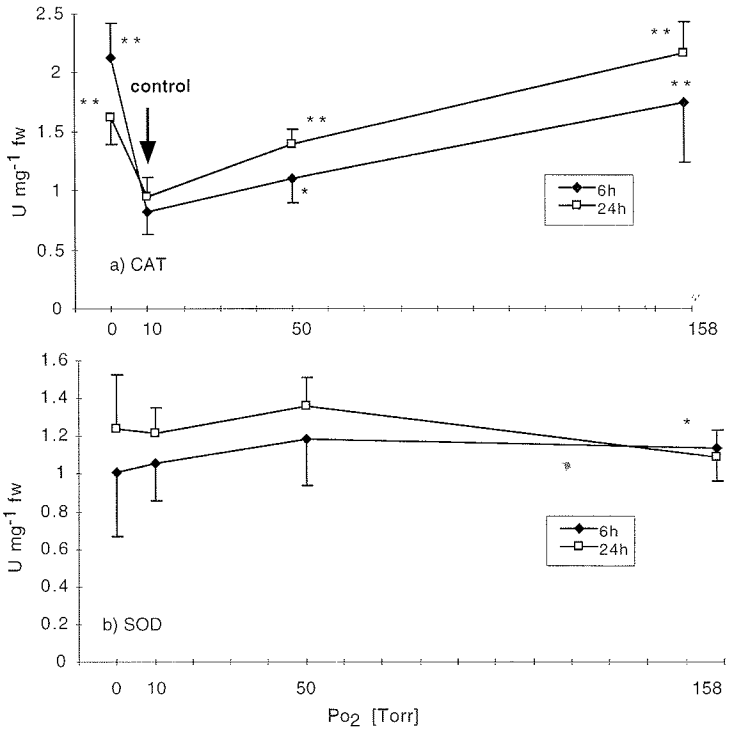

Fig. 3. *Heteromastus filiformis*. AOE activities in whole body extracts versus P_{O_2}. Winter worms, which had previously been acclimated to 10°C over 7 d, were incubated for 6 h and for 24 h at 15°C in a flow-through chamber without sediment. P_{O_2} was adjusted to 0, 10, 50 and 158 torr with P_{CO_2} = constant = 2.3 torr (0.03%). (a) CAT and (b) SOD after 6 h (◆) and 24 h (□) of incubation. Significant differences from control group: $^{**}p < 0.01$, $^{*}p < 0.05$ (n = 5)

while in August temperatures between 20 and 27°C were measured. Activities of both AOE (measured at 25°C) were found to have increased significantly in late spring and in summer as compared to winter values (Table 2).

Difference due to sediment redox state in mudflat versus sandflat sampling sites

A comparison of worms collected from muddy sites with a high loading of fine grained organic material with worms collected from sandflat environments reflects the impact of environmental P_{O_2} and hydrogen sulphide on AOE activities. Sampling was carried out in October 1995 at 2 sites in the Dorum intertidal area. Fig. 5 gives a direct comparison of AOE activities of worms collected at the mudflat and the sandflat locations during different times of the year in 1995.

SOD activity was significantly higher in worms from the sandflat location than in mudflat worms during spring and late summer (Fig. 5b), while there was no difference between SOD activities in worms collected in winter. CAT activities were higher in mudflat than in sandflat worms in winter. However, during spring and late summer no difference of CAT activity could be found between the 2 locations (Fig. 5a).

Catalase activities in polychaete tissues over a tidal cycle in response to increasing hydrogen peroxide concentration in surface water

Hydrogen peroxide concentrations increase in the water of intertidal pools exposed to solar radiation during tidal emersion periods, reaching maximal concentrations of 4 to 5 µmol l^{-1} during summer (Abele-Oeschger et al. 1997). To investigate a possible adaptational response of the hydrogen-peroxide-metabolizing enzyme CAT to increasing H_2O_2 concentrations in surface pool water, a field experiment was run in July 1995. CAT activities were measured in whole worm extracts of the non-ventilating polychaete *Heteromastus filiformis* and in the ventilating polychaete *Hediste* (*Nereis*) *diversicolor*. Natural hydrogen peroxide concentrations and sediment temperatures were recorded throughout the experiment. In addition, specimens of both species were exposed to elevated hydrogen peroxide concentrations under natural conditions, by spiking tide pool water with excess H_2O_2. To do this, a plexiglas corer (∅ 20 cm, 310 cm^2 surface

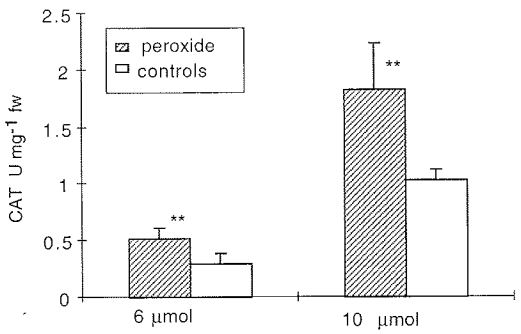

Fig. 4. *Heteromastus filiformis*. CAT activity in specimens after experimental exposure to hydrogen peroxide without sediment. The experiments were conducted in March 1995 with 6 µmol l^{-1} hydrogen peroxide for 6 h and in June 1995 with 10 µmol l^{-1} hydrogen peroxide for 12 h. Experimental conditions were 10°C and normoxia in both cases. Hydrogen peroxide concentrations were continuously controlled throughout the experiments and adjusted when necessary. **Significant difference to control group ($p < 0.01$) (n = 6 to 8)

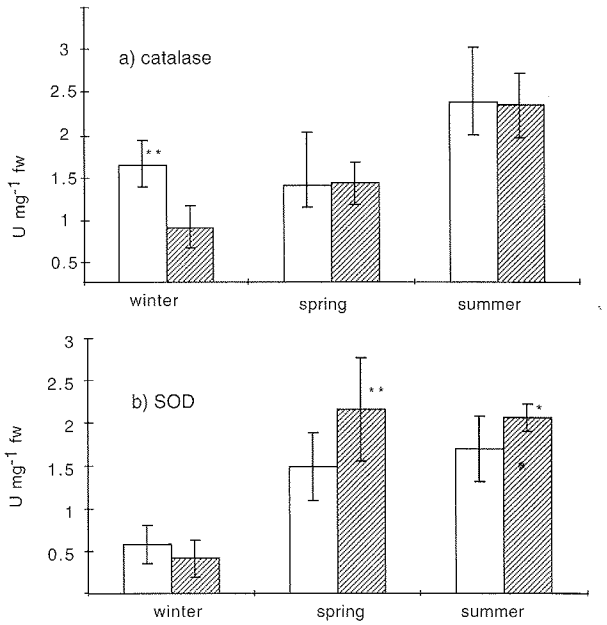

Fig. 5. *Heteromastus filiformis*. AOE activities in whole body extracts from different sampling sites at different seasons. (a) CAT and (b) SOD in whole body extracts measured at 25°C, n = 6 to 8 individuals per group. Open bars: mudflat location with 4 to 5 % organic material of sediment dry weight and H_2S concentrations between 50 and 450 µmol l^{-1} pore water. Hatched bars: sandflat location with 1 % organic content of sediment dry weight and H_2S concentrations between 0 and 150 µmol l^{-1} pore water. Significant difference between mudflat and sandflat individuals: $^{**}p < 0.01$, $^{*}p < 0.05$

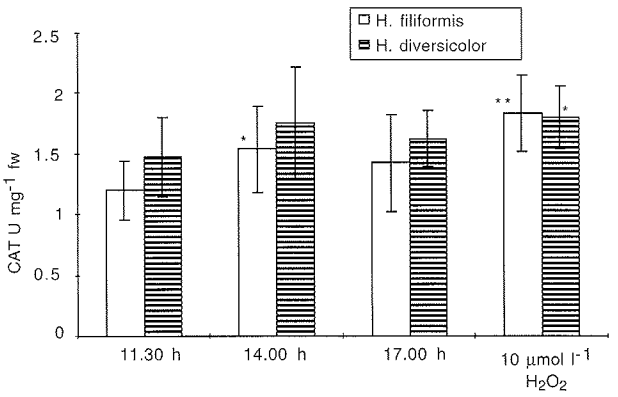

Fig. 6. AOE activities in the polychaetes *Heteromastus filiformis* and *Hediste diversicolor* under *in situ* conditions over a tidal emersion period in July 1995. Additionally, an *in situ* experiment was run in which individuals of both species were exposed to surface water spiked with 10 µmol H_2O_2 l^{-1}. Ambient hydrogen peroxide concentrations in the tide pool water were 3 µmol l^{-1} at 11:30 h (*T*: 26°C) and 4.5 µmol l^{-1} at 14:00 h (*T*: 27°C). **Significant difference to worms collected at 11:30 h ($p < 0.01$) and at 17:00 h ($p < 0.05$). *Significant difference to worms collected at 11:30 h ($p < 0.05$). n = 7 or 8 worms per group (*H. filiformis*) and 5 or 6 (*H. diversicolor*)

area) was inserted into the sediment to 30 cm sediment depth. The enclosed sediment contained polychaetes of both species at high enough densities. The overlying pore water was then spiked with hydrogen peroxide, resulting in a final concentration of 10 µmol l^{-1}. H_2O_2 was measured in the overlying water and readjusted to the experimental concentration when necessary. This experiment was run during 1 ebb tide period, beginning at 11:00 h, with the emersion of the tidal flat and ending at 17:00 h.

CAT activities in both polychaete species from the natural tide pool environment were measured in the morning (11:30 h), and in the early (14:00 h) and late afternoon (17:00 h). The results presented in Fig. 6 show increasing CAT activities in both polychaete species between 11:30 and 14:00 h. This increase was significant only in *Heteromastus filiformis* ($p < 0.05$). No further increase occurred during the afternoon, CAT activities returning to morning levels at 17:00 h. In contrast, the worms incubated with H_2O_2 had significantly higher CAT levels when compared to non-incubated control worms in the morning and at 17:00 h. Again, the effect was more pronounced in *H. filiformis* than in *Hediste diversicolor*.

Influence of the vertical gradient of abiotic parameters in the sediment on Hb distribution and AOE activities in the head and tail end of *Heteromastus filiformis*

To investigate the effect that increasing oxygenation of the sediment surface and the sediment pH gradient have with respect to oxygen transport and potential oxidative stress, a field experiment was conducted during a tidal emersion period in May 1995.

Worms were sampled from an intertidal sandflat at different times after emersion, to see whether AOE and Hb distribution between head and tail end reflect the environmental gradient that establishes itself during tidal emersion. Immediately after collection, worms were sectioned into 3 parts and the middle section discarded. Subsequently, tissues of the head and the tail ends were stored separately on ice for immediate analysis of SOD and CAT activity and Hb content. Tissues of 3 to 4 worms were pooled for each measurement. CAT activity was measured only after 4 h of tidal emersion. SOD and Hb content were measured 0.5, 4 and 5.5 h after emersion of the sandflat.

Fig. 7 shows the development of SOD activity and Hb content per g fw in head and tail ends of *Heteromastus filiformis* during the time course of tidal emersion. Worms collected shortly (30 min) after emersion of the sandflat displayed nearly homogeneous tissue Hb concentration and SOD activity in the head and tail

end, whereas after 4 h of emersion both parameters were significantly higher in the tail end and lower in the head of the worms. At the end of the ebb tide after 5.5 h, the difference between the tail and the head end was more pronounced with respect to SOD activity, but both parameters had reached higher levels in the whole worms. This general increase may thus reflect an artifact due to desiccation of the samples, or water loss of the worms in the sandflat environment due to evaporation at the end of the emersion period (Newell 1979). CAT activity measured after 4 h of tidal emersion showed no difference between the head (1.7 ± 0.56 U mg^{-1} fw) and the tail part (1.39 ± 0.54 U mg^{-1} fw) of the worms.

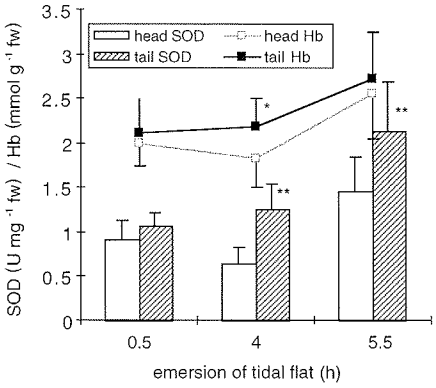

Fig. 7. *Heteromastus filiformis*. SOD activity (bars) and Hb content (lines) in head versus tail parts of specimens over a tidal emersion period. Both parameters were measured in whole body extracts. Data from 1995, n = 7 or 8, each sample comprising tissues from 3 or 4 specimens. Significant differences in tail end versus head end: $^{**}p < 0.01$, $^{*}p < 0.05$

DISCUSSION

The central question of the present study was whether and to what extent the polychaete *Heteromastus filiformis*, an animal adapted to low oxygen and potentially sulphidic environments, experiences oxidative stress in its natural habitat. Which are the abiotic factors eliciting oxidative stress in a basically hypoxic environment? The extent to which oxidative stress occurred was deduced indirectly from the response of enzymatic antioxidants. This is an indirect measure, as an increase of AOE activities is not always observed when oxidative stress occurs (Storey 1996). This may be due partly to new protein synthesis being inhibited or delayed in unfavorable situations (e.g. starvation) when energy reserves can quickly be exhausted (Hand & Hardewig 1996, Kwast & Hand 1996). On the other hand, animals exposed to conditions which potentially involve radical stress can pre-adapt to the situation by generally maintaining elevated tissue antioxidant capacity.

Metabolic rate and P_{O_2}

ROS production in a given tissue is closely related to aerobic metabolic rate (oxygen consumption) and proportional to the amount of mitochondria in tissues (Storey 1996). According to Sohal & Weindruch (1996), between 2 and 3 % of the oxygen consumed by an aerobic cell is converted to $O_2^{\cdot-}$ and H_2O_2. The ability of an organism to regulate aerobic metabolic rates at a given P_{O_2} may thus trigger the extent of oxidative stress an animal will suffer in an environment where the amplitude of P_{O_2} fluctuation can be as high as in the surface of an intertidal sandflat. Animals unable to maintain $\dot{M}_{O_2}$ at a certain level, independent of variable ambient P_{O_2}, are described as 'oxyconforming' (Pörtner et al. 1985) in contrast to 'oxyregulating' species. According to the $\dot{M}_{O_2}/P_{O_2}$ relation as depicted in Fig. 1, *Heteromastus filiformis* is an aerobic oxyconformer and, moreover, is able to to endure very low oxygen conditions for several days (Clough & Lopez 1993). The worms survive prolonged hypoxia by switching to anaerobic energy production with succinate accumulation as confirmed by Oeschger & Vismann (1994) at a P_{O_2} of 1.2 kPa (9 torr) and, moreover, display very low standard metabolic rates (SMR of 0.1 µmol O_2 g^{-1} fw h^{-1}), defined as 'minimum metabolic rate in complete aerobiosis' by Pörtner & Grieshaber (1993). Thus, in accordance with the fact that *H. filiformis* does not oxidize its tube and has its preferred niche in anoxic and often sulphidic sediments, the worms display extremely low basal oxygen uptake. For comparison, this SMR is even lower than that of *Sipunculus nudus* (0.41 µmol O_2 g^{-1} fw h^{-1}; Pörtner et al. 1985), an oxyconforming animal also known for its sluggish behavior. The moderate decline of the $\dot{M}_{O_2}/P_{O_2}$ ratio below the P_c (10 to 15 torr) may, moreover, be indicative of the use of internal oxygen stores in case of limitation of the environmental oxygen supply. Oxygen supply from the internal oxygen stores, i.e. the coelomocyte hemerythrin, at moderate hypoxia has been shown for *S. nudus* by Pörtner et al. (1985). The low P_{50} of *H. filiformis* Hb (0.68 torr; Pals & Pauptit 1979) and the simple morphology of this polychaete favor the view that internal oxygen reservoirs can be used at even lower P_{O_2} values and support aerobic metabolism at the very low P_c.

The more interesting question, however, is: how does *Heteromastus filiformis* deal with high environmental oxygen concentrations? Is there an upper critical P_{O_2} above which the worm's $\dot{M}_{O_2}$ becomes indepen-

dent of environmental P_{O_2}? Our measurements suggest that such a high critical P_{O_2} exists between 10 and 13 kPa (80 and 100 torr).

We also found that keeping the worms under well-aerated conditions resulted in higher mortality, as was also the case in obligate anaerobic meiofauna studied by Wieser et al. (1974). These authors state that in obligate anaerobic nematodes, exposure to normoxia results in abnormally high $\dot{M}_{O_2}$, by far exceeding the normal metabolic rates of the nematodes in their natural low-oxygen environment. Moreover, to these nematodes high P_{O_2} levels proved extremely harmful. Likewise, under natural conditions *Heteromastus filiformis* will never encounter a P_{O_2} of 100 torr at full body length, but if P_{O_2} is high at the sediment surface, only the tail ends of the worms will be exposed.

Elevated oxygen consumption is traditionally considered to indicate elevated ATP turnover in an oxygen consuming tissue. In oxyconformers, the mitochondrial electron transport may be (partly) uncoupled to reduce ATP production at high P_{O_2} levels, as suggested by Pörtner & Grieshaber (1993). Higher metabolic rates will enhance ROS liberation and increase the risk of ROS mediated tissue injury, if activities of ROS scavenging enzymes are not high enough to outbalance ROS production (Sohal & Weindruch 1996, Storey 1996). Therefore, higher respiration rates also imply enhanced ROS release from *Heteromastus filiformis* mitochondria at high P_{O_2} levels. We hypothesize, however, that the worms prefer hypoxic environments. When P_{O_2} rises, e.g. during photosynthetic activity at the surface, a transient increase of oxygen consumption, induced by uncoupling of the ATP synthesis, will support a faster reduction of P_{O_2} in the confined environment of the upper oxidized tube ending. The worms would then be able to return to lower $\dot{M}_{O_2}$.

Response of AOE activities to changes in the abiotic environment

CAT and SOD response to P_{O_2}, temperature and H_2O_2

Higher AOE activities in summer versus winter *Heteromastus filiformis* control groups (Fig. 2) argue for a long-term effect, involving seasonal adaptations, i.e. increased expression of AOE genes during spring and summer. By contrast, short-term exposure (14 d) to elevated P_{O_2} and higher (winter worms) or lower (summer worms) temperatures in laboratory experiments elicited only minor changes in SOD activities (Figs. 2 & 3b). A pronounced short-term response to alternating P_{O_2} was, however, found with CAT, which increased significantly upon exposure to anoxia and normoxia (21 kPa; Fig. 3a). Thus, the existing SOD tissue concentration was obviously sufficient to trigger higher CAT activity via higher internal H_2O_2 production. Additionally, a spontaneous, non-enzymatic conversion of oxygen radicals, leaking from *H. filiformis* mitochondria during high metabolic activity, and even more so when the electron transporters of the respiratory chain become reduced under anoxia (Storey 1996), provide a potential explanation for the observed increase of CAT activity.

CAT activity in *Heteromastus filiformis* was also significantly induced upon experimental exposure to external H_2O_2 (Fig. 4). Peroxide induced increase of CAT activities in polychaetes from intertidal flats has previously been reported by Abele-Oeschger et al. [1994; *Nereis* (*Hediste*) *diversicolor*] and Buchner et al. (1996; *Arenicola marina*). In the present study, however, we investigated the effect of external hydrogen peroxide on a worm which does not oxidize its burrow, thus minimizing the contact with H_2O_2-loaded surface water. The measurements of AOE activities in specimens from the sandflat sampling site at different times of the year showed a clear increase in CAT activity in spring and summer compared to winter worms, which is related to the elevated peroxide accumulation in intertidal surface waters.

A much clearer picture of the peroxide effect on CAT resulted from measurements of CAT activities in worms throughout a tidal emersion period. H_2O_2 accumulation in intertidal surface waters has been shown to be a fast process which on sunny days can yield more than 300 nmol l^{-1} H_2O_2 net production h^{-1} around noon. Later in the afternoon, peroxide decomposition becomes the dominating process, leading to declining concentrations in surface pool waters (Abele-Oeschger et al. 1997). The CAT activities in tissues of *Heteromastus filiformis* and *Hediste diversicolor*, the second polychaete studied, followed exactly the same pattern. Like the peroxide levels in intertidal pool water, CAT activities reached a maximum at 14:00 h, and showed a tendency to decline again at 17:00 h. Individuals of both species that were exposed to H_2O_2 spiked surface water had higher levels of CAT activity than worms exposed to natural peroxide accumulation (Fig. 6). These experiments show how environmental ROS formation can induce CAT activity in infaunal polychaetes under *in situ* conditions, irrespective of the individuals' ventilatory activity. It remains unclear whether the increase in CAT activity is due to de novo protein synthesis. More likely, previously inactive CAT dimer subunits form the active tetramer, a process triggered by inflowing H_2O_2 (Halliwell & Gutteridge 1985), which enables the enzyme to perform short-

term responses. In contrast, SOD activity changes on a seasonal time scale only.

SOD and CAT response to hydrogen sulphide

Exposing worms to hydrogen sulphide under oxic as well as under anoxic conditions, we found lower SOD levels under anoxic conditions. Lower SOD activities in mudflat worms compared to sandflat specimens during spring and summer may therefore be related to anoxia and possibly to higher H_2S concentrations in the mudflat environment.

Although both types of AOE are generally known to be inhibited by H_2S (SOD only in the CuZn form), in *Heteromastus filiformis* anoxia with sulphide caused no further depletion of SOD and only a slight reduction of CAT activity as compared to anoxia without sulphide. Oeschger & Vismann (1994) found a very high sulphide inhibition constant (compared to less sulphide tolerant invertebrates) for *H. filiformis* CAT *in vitro* (790 µmol H_2S l^{-1}), which they related to the necessity for sulphide insensitive AOEs in order to scavenge H_2O_2 and oxygen radicals generated during the process of mitochondrial sulphide oxidation, as has been suggested by Tapley (1993a, b). Thus, adaptation to sulphidic and anoxic environments may also include acquiring sulphide insensitive forms of AOEs (Abele-Oeschger 1996).

Cumulative effect of abiotic factors and the sedimentary gradient on SOD activity in the head and tail end of *Heteromastus filiformis*

Taking into consideration the various abiotic factors and their impact on *Heteromastus filiformis* AOE activities under experimental and field conditions, a cumulative effect of the abiotic sedimentary gradient, building up over the tidal emersion period, is to be expected. Significantly higher SOD activities in the tail end compared to the head end after 4 h of tidal emersion are related to the higher P_{O_2} at the sediment surface, which in turn is due to microalgal photosynthetic activity. It also reflects the importance of the gradient, as SOD did not increase upon exposure of whole worms to 21 kPa P_{O_2} in the laboratory experiment (Fig. 3b). As Hb is also concentrating in the tail end, the SOD activity is presumably either an integral part of the polychaetes' Hb (Abele-Oeschger 1996) or is contained within the coelomocytes.

Overall, the experiment shows how *Heteromastus filiformis* and other invertebrates colonizing intertidal sand- and mudflats experience fluctuations in oxidative stress not only from temporal variations of P_{O_2}, but also from spatial gradients, where H_2S may accumulate around the head end, while at the same time the tail end is subjected to hyperoxia at the sediment surface. An additional effect of the pH gradient on AOE, especially SOD, activity *in situ* could not be confirmed in our experiments on *H. filiformis*.

CONCLUSION

In conclusion we would like to emphasize that *Heteromastus filiformis* in its sediment microhabitat is apparently in need of considerable tissue antioxidant capacity. In fact, the AOE levels measured during the course of this study were in the range of those measured in ectothermal, air-breathing vertebrates (Storey 1996). This is surprising, because these polychaetes display extremely low standard metabolic rates and, moreover, minimize their contact with oxygenated seawater, owing to their non-ventilating mode of oxygen uptake.

However, we have identified several factors in the abiotic environment of *Heteromastus filiformis*, such as temperature, P_{O_2}, hydrogen peroxide and H_2S, which may either directly cause oxidative stress (H_2O_2), elicit internal ROS liberation when critical alterations occur (T, P_{O_2}), or modulate AOE activity (H_2S), thereby enhancing or mitigating oxidative stress. Comparatively high levels of AOE activities as seen in *H. filiformis* whole tissue extracts can be seen as an adaptation of the antioxidant system to cope with potential oxidative stress caused by fluctuations of these abiotic parameters, or by a combination of stress factors (anoxia and H_2S, anoxia and H_2O_2, etc.). Short-term adjustments of AOE activities over tidal emersion periods can help to reduce elevated stress due to UV induced hydrogen peroxide accumulation in surface waters during summer ebb tides. These rapid adjustments involve ROS induced activation of inactive enzyme subunits (CAT) or the shift of SOD activity to the tail end of the polychaete which is exposed to higher P_{O_2} and H_2O_2 at the sediment surface. As a sulphide tolerant species, *H. filiformis* has acquired sulphide insensitive forms of CAT and SOD to neutralize ROS produced during mitochondrial sulphide oxidation (Oeschger & Vetter 1992).

Acknowledgements. We thank the 'Nationalparkhaus Niedersächsisches Wattenmeer' at Dorum-Neufeld and especially Dipl. Biol. W. Menger for supporting this work by providing laboratory space during the field studies. We thank Professor W. Wieser and 2 anonymous referees for their constructive revision of the paper. This study was funded by a research grant from the Deutsche Forschungsgemeinschaft (DFG, Ab 64/1-3). This is Alfred Wegener Institute Publication No. 1363.

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Editorial responsibility: Otto Kinne (Editor), Oldendorf/Luhe, Germany

Submitted: June 18, 1997; Accepted: December 12, 1997
Proofs received from author(s): February 9, 1998