

Sulfide detoxification and tolerance in *Halicryptus spinulosus* (Priapulida): a multiple strategy

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ABSTRACT: The detoxification potential of *Halicryptus spinulosus* in the presence of sulfide was studied. The worms are able to cope with considerable amounts of sulfide. They benefit from several survival strategies for controlling sulfide dose and detoxifying sulfide that enters the body. When oxygen and sulfide occur concurrently in the environment sulfide can either initially be excluded or internal concentration kept low by the animals. Non-toxic thiosulfate, the main detoxification product, accumulates up to $3856 \pm 997 \mu\text{M}$ in the hemolymph. Sulfide has an overriding influence on the aerobic metabolism and imposes anaerobiosis even though oxygen is available, i.e. sulfide-induced anaerobiosis. Succinate, an indicator of anaerobic metabolism, accumulates during persistent oxic sulfide incubation to $12.3 \pm 2.3 \mu\text{mol ml}^{-1}$ in hemolymph and to $3.0 \pm 1.3 \mu\text{mol g}^{-1}$ fresh mass in body wall after 10 d. During anoxic sulfide exposure, the animals make use of graded strategies. Some sulfide is immobilised in an external barrier due to iron-sulfide formation. The iron-sulfide compound results in a conspicuous blackening of the animal and its blood. This blackening is fully reversible upon receiving oxygen again. Detoxification to thiosulfate, depending on the oxygen storage capacity of the hemolymph, amounted to only a minor level, with a maximum of $172 \pm 49 \mu\text{M}$ in the hemolymph after 1 d of incubation. Sulfide also binds to a presently unknown hemolymphic factor. This binding may temporarily protect mitochondria, a main location of sulfide detoxification. The mitochondrial enzyme for aerobic metabolism, cytochrome c oxidase, is inhibited by low sulfide levels *in vitro* (K_i $1.05 \pm 0.22 \mu\text{M}$), and has a much higher *in vivo* resistance in whole animals. Catalase, another sulfide sensitive enzyme, has a K_i of ca $200 \mu\text{M}$ *in vitro* and is unaffected *in vivo*. Internal sulfide concentration in the hemolymph increases ca 3-fold ($570 \pm 298 \mu\text{M}$) over external levels during long-term exposure experiments with $200 \mu\text{M}$ sulfide. The survival in spite of such an elevated amount of internal sulfide accumulation is unprecedented for a marine invertebrate without endosymbionts. The worms can passively outlast raised environmental sulfide concentrations by their high capacity for anaerobic metabolism.

INTRODUCTION

Recent studies stress the importance of sulfide in a variety of marine environments other than deep-sea hydrothermal vents (Bagarinao & Vetter 1989, 1990, Cary et al. 1989b, Schiemer et al. 1990, Llansó 1991, Ott et al. 1991, Vismann 1991a, Völkel & Grieshaber 1992).

Although sulfide has been known to be abundant in marine sediments, this highly toxic compound has usually been disregarded as a factor influencing the distribution of soft bottom benthic macrofauna. Exposure and impact of sulfide on marine invertebrates have

attained considerable attention during the last years due to the discovery of the spectacular deep-sea hydrothermal vents (for reviews e.g. Somero et al. 1989, Fisher 1990), which has stimulated intensive research on this topic. The well-aerated sulfide-containing deep-sea vents are mostly inhabited by flourishing populations of marine macrofauna with sulfide-oxidising endosymbiotic bacteria (e.g. Cavanaugh et al. 1981, Powell & Somero 1983, Jones 1985, Fisher 1990).

Sulfide-rich marine soft sediments have different features. Constant anoxic sulfidic sediments like those of the deep basins of the Black Sea and the Baltic Sea are without higher organisms and only the borders of these areas are colonised by some invertebrates. A different situation is encountered in areas with sea-

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sonal fluctuations of oxygen availability and subsequent sulfide development. Sulfide is produced in the anoxic sediment layers. When oxygen becomes limited the anoxic layer with the sulfide expands upwards. Such biotopes are either populated by opportunistic species with little or no capacity for anaerobic metabolism or by species with an extraordinary anaerobic capacity and sulfide resistance. Opportunistic species are able to survive only during intermittent periods when oxygen is available and sulfide is absent. With deteriorating conditions these populations will be extinguished. In areas like those of the Western Baltic only a few species are able to resist frequent adverse environmental conditions. Earlier studies on such species showed a high anaerobic capacity (Theede et al. 1969, Theede 1984, Oeschger 1990). The importance of anaerobiosis as a strategy to avoid sulfide toxicity has yet to be intensively addressed.

Halicryptus spinulosus, a priapulid worm and one of those few species able to persist in anoxic soft sediments of the Western Baltic, is frequently exposed to elevated sulfide levels. It is an important faunal component with high abundances and it survives for extended periods at environmental anoxia (Weigelt & Rumohr 1986). Previous studies on *H. spinulosus* dealt with its anaerobic capacity (Oeschger 1990, Oeschger & Storey 1990), energetic studies (Oeschger et al. 1992), and with histological and ultrastructural peculiarities of sulfide impact on the animals (Oeschger & Janssen 1991, Janssen & Oeschger 1992).

The objective of this paper was to study sulfide detoxification and tolerance in *Halicryptus spinulosus*. We conducted studies on whole animals to look at the oxic and anoxic sulfide detoxification potential and to assess the involvement of an anaerobic metabolism. Further points of interest were the effects of sulfide exposure on enzymes. Shipboard studies on the actual sulfide contents in worms and the sediment were performed to complete this study.

MATERIAL AND METHODS

Sampling. *Halicryptus spinulosus* were dredged during several cruises between summer 1990 and winter 1991/92 from muddy soft sediments at a water depth of 20 m in Kiel Bight, Western Baltic Sea. Specimens were transported live in a cooler to the Scripps Oceanographic Institution, La Jolla, California, USA, in summer 1990. They were kept unfed in aerated seawater at $9.5 \pm 0.5^\circ\text{C}$ and 22‰ S in original sediment. Prior to experiments worms were kept at least 5 d in aerated seawater without sediment.

During 1990 to 1992 some worms were analysed for sulfide contents directly after capture aboard the

research vessel. In November 1991 a specially designed light-weight gravity sediment corer (Meischner & Rumohr 1974) was used from shipboard to obtain pore water from undisturbed sediment cores (diameter 80 mm) of the sampling area. Sediment was centrifuged immediately aboard ship and analysed for sulfide contents after Cline (1969), using the 1 to 40 μM range of reagents.

Oxic sulfide incubation. A continuous flow-through system with a chamber volume of about 1.5 l was used. A peristaltic pump delivered supersaturated (107%) seawater ($10 \pm 0.5^\circ\text{C}$, 22‰ S) at a constant flow rate of about 800 ml h^{-1} . A sulfide stock solution (3.2 mM, pH adjusted with HCl to 7.0) was delivered by another peristaltic pump and mixed with seawater before entering the incubation chamber to obtain the experimental concentration. During the experiments oxygen and sulfide concentrations were regularly monitored. For oxygen measurements a modified Winkler procedure (after Ingvorsen & Jørgensen 1979) had to be used due to sulfide interaction with the conventional determination: sulfide was precipitated with 2 N ZnSO_4 in the presence of 1 N NaOH and oxygen could then be reliably estimated in the overlaying seawater. Oxygen concentrations were mainly in the range of 90 to 97%, referred to as 'oxic incubation'. Sulfide concentrations were in the range of $200 \pm 40 \mu\text{M}$ and pH in the chamber was between 7.84 and 7.98. In this paper the term sulfide refers to total sulfide (H_2S , HS^- and S^{2-}) unless otherwise stated.

Anoxic sulfide incubation. For anoxic sulfide exposure experiments (200 μM), worms were incubated in an airtight experimental chamber containing 500 ml seawater ($9.5 \pm 0.5^\circ\text{C}$, 22‰ S). Sulfide levels were obtained by adding $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ crystals to nitrogen or argon-bubbled oxygen-free seawater to a given final concentration of ca 200 μM and pH was adjusted to 8.0 with HCl. After sealing the chamber, an argon atmosphere was introduced to cover the surface additionally against oxygen. During exposure experiments triplicate water samples were taken and the sulfide concentration was measured by the methylene blue method (Cline 1969). Due to oxidation during incubation, H_2S concentrations were readjusted daily to the intended level. This happened particularly during the first 3 to 4 d of incubation, while sulfide concentration was nearly unchanged afterwards. To avoid the accumulation of toxic metabolites, the seawater was changed every 3 to 4 d. For anoxic sulfide exposure we chose a concentration of 200 μM since this is a realistic concentration that the worms encounter during long-term environmental oxygen deficiency.

Enzyme assays. **Cytochrome c oxidase:** Activity of cytochrome c oxidase (E.C. 1.9.3.1) in freshly sampled body wall tissue and hemolymph of *Halicryptus*

spinulosus was measured after Hand & Somero (1983). *In vitro* sulfide inhibition experiments were performed on homogenates from unexposed worms using different sulfide concentrations to calculate the 50% inhibition constants (K_i or I_{50} -value). Activity of cytochrome c oxidase was also assayed in specimens exposed to 200 μM sulfide for 8 d (22‰ S, $9.5 \pm 0.5^\circ\text{C}$).

Catalase: Activity of catalase (E.C. 1.11.1.6) was determined according to Aebi (1985). Samples were homogenised in 40 mM potassium phosphate buffer. The homogenate was assayed in potassium phosphate buffer (50 mM, pH 7.0) containing H_2O_2 as substrate (35 μl of a 30% H_2O_2 stock solution diluted with 10 ml of phosphate buffer). From this solution, 350 μl were added with a varying volume of phosphate buffer and homogenate to a final volume of 1 ml. The initial absorbance at 240 nm was in the range of 0.500, and time needed for a decrease from 0.450 to 0.400 was recorded to calculate the activity of catalase. Since this reaction exhibits abnormal kinetics, the rate constant of the first order reaction [k , according to the equation $k = 1/t \cdot \ln(E_0/E)$] has to be converted to units. One unit is defined as the decomposition of 1 μmol H_2O_2 min^{-1} , while the concentration of H_2O_2 decreases from 10.3 $\mu\text{mol ml}^{-1}$ ($\text{abs}_{240} = 0.450$) to 9.2 $\mu\text{mol ml}^{-1}$ ($\text{abs}_{240} = 0.400$).

Bovine liver catalase (Sigma) was used as a reference standard. Sodium azide (1 mM) was used as an inhibitor of catalase. *In vitro* inhibition of different concentrations of sulfide were assayed and I_{50} -values were calculated.

Succinate. The anaerobic metabolite succinate was measured using the standard enzymatic determination after Beutler (1985).

Chromatography of sulfur compounds in body wall tissue and hemolymph. Determination and quantification of sulfur compounds was done by reversed-phase high-performance liquid chromatography (HPLC) (Fahey & Newton 1987, Vetter et al. 1989), with monobromobimane (mBBr), a synthetic molecule that covalently binds reduced sulfur compounds (thiols) and makes them fluorescent. The term 'thiol' refers to sulfhydryl compounds which react with mBBr. In this paper the term is extended to sulfite, sulfide and thiosulfate. Samples were run on a computer-controlled Kontron HPLC system, excitation wavelength of the detector was set at 350 nm, emission wavelength on 480 nm. Quantification was achieved by comparing the peaks with authentic standards: fluorescence of the sulfur compounds in the samples was measured against standard mixtures of sulfide, thiosulfate, sulfite, cysteine, and glutathione. To ascertain that the peaks detected were sulfur compounds, a subsample was derivatized with non-fluorescent 2-dipyridyl-sulfide (PDS), which also binds sulfur species but prevents

mBBr binding. After the experiments, body wall tissue was immediately homogenised in an ice-cold HEPES-buffer (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, 200 mM, pH 8.0) containing the mBBr and prepared for further HPLC analysis (Vetter et al. 1989). Hemolymph was prepared without pretreatment. Samples were either directly analysed or stored at 4°C . Storage at 4°C had no significant effect on content of sulfur compounds (Fahey & Newton 1987).

In some experiments oxidation of sulfide and conversion to other metabolites was studied using radiolabelled Na_2^{35}S (Amersham). The HPLC system was able to detect fluorescence and radioactivity of ^{35}S simultaneously, the latter by a flow-through in-line scintillation counter (Vetter et al. 1989).

Hemolymph binding. To detect the possible presence of a sulfide binding component in the hemolymph of *Halicryptus spinulosus*, we used ^{35}S -radiolabelled sulfide in connection with a dialysis chamber. Hemolymph samples from 4 to 8 unexposed oxic controls, or anoxic worms which had been exposed for 10 d to a sulfide concentration of 200 μM , were placed in plugged cylinders of dialysis tubing (molecular weight cut off: ca 12 kDa) using a Hamilton syringe (final sample volume: 1 ml). The dialysis bag was placed in a water-jacketed stirring reservoir of 12 ml buffer volume (seawater of 22‰ S, buffered with 10 mM Tris, pH 7.5). Control samples without hemolymph consisted of buffered seawater and were also placed in separate dialysis bags. Cold and radiolabelled sulfide was added to the outer buffer to a final concentration of 200 μM in a ratio 3:1. The buffer was moderately degassed prior to use to prevent an immediate spontaneous oxidation of the sulfide. Repeated sampling and scintillation counting of the ^{35}S radioactivity in the samples and the control bag showed the diffusion and final equilibrium. The measure for binding capacity was the difference in counts between buffer control and hemolymph samples.

Isolation and respiration of mitochondria. For one mitochondrial isolation, ca 15 to 20 worms (8 to 9 g fresh mass) were homogenised. The body wall of freshly dissected worms was cleansed of adhering coelomic fluid and other tissues, rinsed in isolation buffer, and cut in coarse pieces. This suspension was homogenised using a 10 to 15 s burst (9500 rpm) of an Ultra Turrax tissue homogenizer, and further homogenised in a Potter-Elvehjem homogenizer with 3 to 5 passes of a teflon pestle (final volume: ca 8 to 9 g fresh mass per 30 ml isolation buffer). The isolation buffers consisted of 350 to 500 mM glycine, 170 to 200 mM sucrose, 2 mM EGTA [ethylene glycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid], 2 mM EDTA (ethylene diamine-N,N,N',N'-tetraacetic acid), 25 to 100 mM KCl, 20 mM HEPES, 0.2% BSA (bovine

serum albumin, essentially fatty acid free), pH 7.4. The resulting preparation was centrifuged at 600 *g* for 6 min to remove cellular debris. A second spin at 13 500 *g* for 15 min was used to pellet the mitochondria of the supernatant of the first spin. The upper layer of the resulting pellet was resuspended in 0.5 to 1 ml of isolation buffer. Any further purification steps lead to significantly reduced or no mitochondrial activity and were omitted. Activity of GDH (glutamate dehydrogenase) was used as a mitochondrial marker enzyme. Mitochondrial preparations usually contained 2 to 4 mg protein ml⁻¹ (determined by the Biuret method).

Assays of mitochondrial respiration were monitored at 13 °C in an oxygen respiration chamber described by O'Brien & Vetter (1990) to determine the respiratory control ratio (RCR) (Estabrook 1967). The respiration medium was the same as used for isolation plus up to 10 mM potassium phosphate and 7 mM 2-oxoglutarate. For oxygen respiration measurements of the mitochondria, a gold-coated sulfide-insensitive Clark type oxygen electrode (Eschweiler, Kiel, Germany) was used.

RESULTS

Environmental sulfide

Sediment

In Fig. 1 the sulfide concentration in the pore water of the sampling location in November 1991 is shown (Western Baltic Sea: 54° 38.1' N, 10° 20.5' E; water depth 21 m). *Halicryptus spinulosus* is found down to 30 cm sediment depth. Results indicate that the worms can be exposed to a concentration of up to 665 μM sulfide in the pore water. On earlier cruises the directly measured sulfide concentrations in samples of pooled pore water from 0 to 5 cm sediment depth were: 30 to 49 μM (27 November 1990); 4 to 29 μM (27 February 1991), and 2 to 16 μM (2 July 1991).

Worms

In Table 1 sulfide and thiosulfate concentrations in worms from different cruises are compiled. Hemolymph samples were taken on shipboard immediately after obtaining the worms to determine actual *in situ* values. Some *Halicryptus spinulosus* were subsequently kept in the laboratory in aerated seawater without sediment to monitor the course of the sulfide and thiosulfate concentration. On some occasions nearly all dredged *H. spinulosus* were black, reflecting sulfide exposure. During collection, both pale to brownish-looking and black worms were caught, indi-

cating a patchy distribution of sulfide in their habitat. Internal sulfide concentrations decreased during the laboratory incubation and thiosulfate was not detectable, with one exception showing small amounts.

Sulfide incubation experiments

Whole animals

Halicryptus spinulosus usually has a pale to dark brown colour when living in oxidised sediment without sulfide. During the experiments some individuals started to turn black on the surface of their body wall after only about 1 h exposure to 200 μM sulfide containing anoxic seawater. The blackening intensified after 2 h and the body wall of all worms was completely black after 5 to 6 h of incubation, whereas the hemerythrin-containing hemolymph turned completely black after ca 3 d of incubation.

On one occasion we observed that the process of regaining the usual pale brown colour after being transferred to aerated seawater started from the middle of the body wall, and the worms regained their normal colour within 1 to 2 min. Such a rapid colour change might indicate the involvement of an enzyme-mediated mechanism in reversing the iron-sulfide interaction. During oxic sulfide incubation (200 μM) about 50 % of the worms turned black after ca 3 d, but not as intensely as during anoxic sulfide incubation.

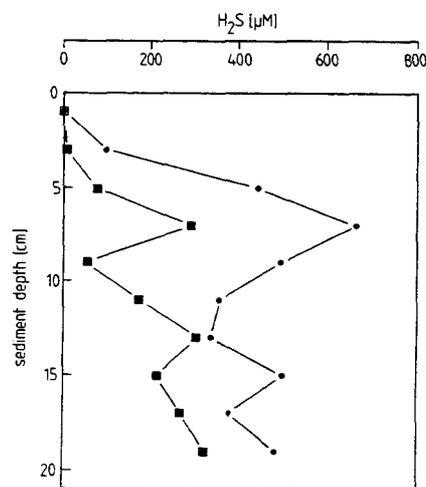


Fig. 1. Sulfide concentration in pore water (in μM) from the sampling location of *Halicryptus spinulosus* in the Western Baltic Sea in November 1991 determined by the methylene blue method. (●) Core 1; (■) Core 2

Sulfide concentration in whole animals was $6.6 \pm 4.3 \text{ nmol g}^{-1}$ fresh mass ($n = 5$), while thiosulfate levels were $1.02 \pm 1.39 \text{ nmol g}^{-1}$ fresh mass ($n = 5$).

Body wall

During oxic sulfide incubation ($200 \text{ }\mu\text{M}$), sulfide started to accumulate in body wall tissue of *Halicryptus spinulosus* from an initial $0.028 \pm 0.014 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 4$) in control worms to $0.106 \pm 0.164 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 4$) after 1 d of exposure. After 10 d, sulfide concentration rose to $0.201 \pm 0.072 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 6$) (Fig. 2a). Thiosulfate, an oxidation product of sulfide, increases strongly after 1 d of incubation from an initial $0.07 \pm 0.03 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 4$) to $0.275 \pm 0.143 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 5$), reaching a maximum of $0.779 \pm 0.14 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 5$) after 4 d. Thiosulfate concentration tended to decrease to $0.422 \pm 0.17 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 5$) at the end of the experiments (Fig. 2b).

In anoxic sulfide incubations ($200 \text{ }\mu\text{M}$), a large accumulation of sulfide was measured in worms. Values increased from $0.079 \pm 0.037 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 6$) in controls to $0.644 \pm 0.286 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 14$) after 10 d. We assessed a possible effect of the mucus covering the outer body wall on sulfide values in body wall tissue. Some samples of a 10 d anoxic incubation ($200 \text{ }\mu\text{M}$) were sonicated for 30 s to strip off the mucus prior to analysis. Values of $0.487 \pm 0.398 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 8$) for sonicated tissues tended to be

smaller compared to untreated individuals. After sonication worms were almost pale; their creamy looking gonads and the black hemolymph became visible.

Only minor amounts of thiosulfate were produced after 1 d of incubation ($0.036 \pm 0.027 \text{ }\mu\text{mol g}^{-1}$ fresh mass, $n = 5$) and tended to accumulate at the end of the 10 d experiments ($0.092 \pm 0.045 \text{ }\mu\text{mol g}^{-1}$ fresh mass, $n = 14$). During anoxic sulfide incubations, thiosulfate concentrations amounted to only a minor fraction of that found during oxic sulfide incubation. Sulfite (SO_3^{2-}), GSH (reduced glutathione) and cysteine were also measured. Sulfite and cysteine were detectable only in traces in control individuals as well as in incubated specimens (data not shown). The concentration of GSH did not differ significantly during the course of the experiments. Concentrations ranged from $0.462 \pm 0.17 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 7$) in controls to $0.40 \pm 0.118 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 6$) in oxic incubated specimens after 10 d, and to $0.448 \pm 0.114 \text{ }\mu\text{mol g}^{-1}$ fresh mass ($n = 14$) after 10 d of anoxia.

Hemolymph

In a short-term experiment $100 \text{ }\mu\text{l}$ of hemolymph was incubated with ^{35}S -sulfide for 6 h. Analysis showed some ^{35}S -sulfate formation due to chemical oxidation, suggesting that sulfate did not participate in sulfide detoxification. Double radiolabelled thiosulfate (^{35}S - $^{35}\text{SO}_3^{2-}$) was identified as the detoxification product.

At the beginning of oxic sulfide incubation ($200 \text{ }\mu\text{M}$),

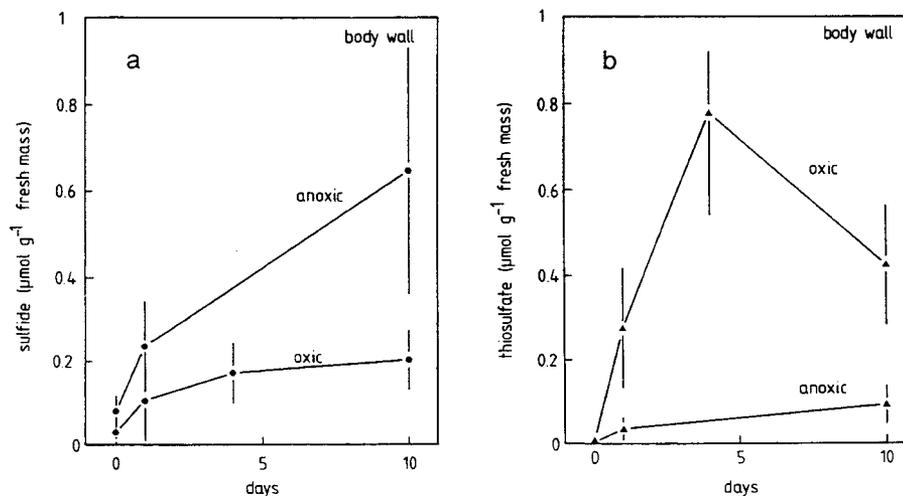


Fig. 2. *Halicryptus spinulosus*. Concentrations (\pm SD) of (a) sulfide and (b) thiosulfate in body wall tissue during oxic and anoxic sulfide incubation after 1, 4 and 10 d at an external sulfide concentration of $200 \text{ }\mu\text{M}$ in seawater ($9.5 \pm 0.5 \text{ }^\circ\text{C}$, 22‰ S). Values are in $\mu\text{mol g}^{-1}$ fresh mass. Data are means of 5 to 14 individuals

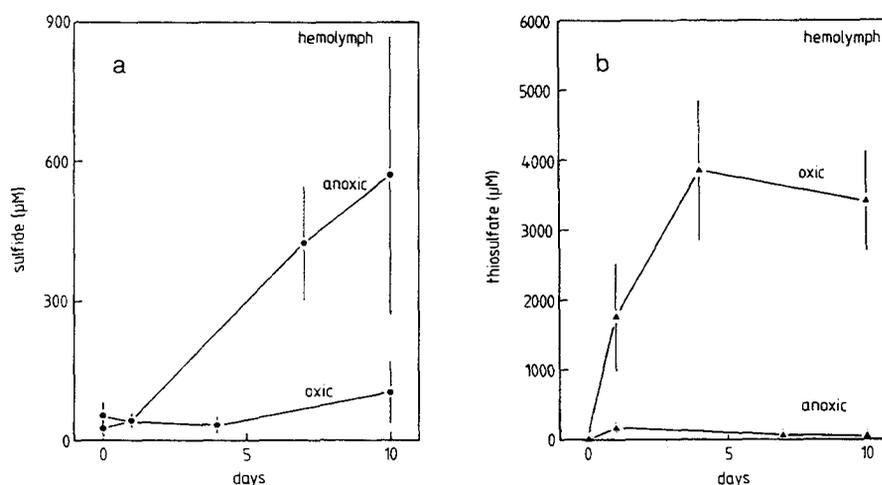


Fig. 3. *Halicryptus spinulosus*. Concentrations (\pm SD) of (a) sulfide and (b) thiosulfate in hemolymph during oxic and anoxic sulfide incubation after 1, 4, 7, and 10 d of an external sulfide concentration of 200 μ M in seawater ($9.5 \pm 0.5^\circ\text{C}$, 22‰ S). Values are in μ M. Data are means of 5 to 18 individuals

Halicryptus spinulosus was able to keep the sulfide from entering the hemolymph. In the course of the experiments, sulfide concentration varied between $53 \pm 29 \mu\text{M}$ ($n = 5$) in controls to $34 \pm 17 \mu\text{M}$ ($n = 5$) after 4 d of incubation, but was $104 \pm 67 \mu\text{M}$ after 10 d ($n = 6$) (Fig. 3a). Thiosulfate increased from $6 \pm 13 \mu\text{M}$ ($n = 5$) in controls to $1756 \pm 781 \mu\text{M}$ ($n = 5$) after 1 d of incubation, reaching a maximum of $3856 \pm 997 \mu\text{M}$ ($n = 5$) at 4 d, while it tended to decrease slightly to $3410 \pm 718 \mu\text{M}$ ($n = 6$) after 10 d (Fig. 3b).

The hemolymph exhibited a different characteristic during anoxic sulfide exposure (200 μM). After 1 d of incubation sulfide concentration ($43 \pm 15 \mu\text{M}$, $n = 5$) did not differ from controls ($26 \pm 18 \mu\text{M}$, $n = 14$). After 7 d the sulfide level had increased to 424 ± 122 ($n = 6$), and reached $570 \pm 298 \mu\text{M}$ ($n = 18$) at the end of the experiments. Thiosulfate concentration accumulated from $0.4 \pm 0.7 \mu\text{M}$ ($n = 13$) in controls to a peak of $172 \pm 49 \mu\text{M}$ ($n = 3$) after 1 d of anoxic incubation and was $56 \pm 29 \mu\text{M}$ after 10 d ($n = 16$).

Sulfite and cysteine were only present in traces and were not involved in sulfide detoxification (data not shown). GSH concentrations showed no variation. The level in hemolymph was $247 \pm 184 \mu\text{M}$ ($n = 11$) in controls and $257 \pm 135 \mu\text{M}$ ($n = 18$) after 10 d of anoxic incubation. In oxic sulfide experiments GSH concentrations were $232 \pm 74 \mu\text{M}$ ($n = 6$) after 10 d.

Sulfide binding in the hemolymph

Freshly sampled hemolymph of *Halicryptus spinulosus* was tested for the presence of a sulfide binding factor by means of equilibrium dialysis against ^{35}S -

radiolabelled sulfide. The hemolymph has a binding factor which concentrates sulfide nearly 2-fold over the external sulfide concentration (Fig. 4). Hemolymph from specimens previously exposed to sulfide (200 μM), and specimens kept in sulfide-free seawater prior to the experiment, bound radiolabelled sulfide, suggesting that some unlabelled sulfide in the hemolymph of exposed worms is exchanged for radiolabelled sulfide during dialysis.

The formation of a black fraction of the hemolymph during sulfide incubation occurs in the erythrocytes, since spinning of sulfide-incubated hemolymph resulted in a black pellet and clear supernatant. Spinning of either untreated hemolymph or lysed erythrocytes by ultrasonification with Centricon molecular weight cut-off membranes (10 kDa) indicated that the black component was associated with the fraction above 10 kDa, since the black fraction did not pass the membrane.

Succinate

During oxic sulfide incubation (200 μM) *Halicryptus spinulosus* accumulated only small amounts of succinate in the beginning of the experiments (Fig. 5). After 4 d, levels of succinate increased significantly in hemolymph ($11.3 \pm 5.1 \mu\text{mol ml}^{-1}$) and body wall ($2.3 \pm 0.9 \mu\text{mol g}^{-1}$ fresh mass) with a trend of further accumulation after 10 d of incubation. This indicated that the worms could not maintain aerobic metabolism during long-term oxic sulfide exposure. Controls kept aerated before and during the experiments had succinate levels in the same range (Fig. 5).

Table 1. *Halicryptus spinulosus*. Concentration (\pm SD) of sulfide and thiosulfate in hemolymph of worms immediately sampled on shipboard during different cruises, or after various periods in aerated seawater without sediment in the laboratory. Values of sulfur compounds are given in μM ; no. of samples in parentheses. tr: traces; nd: not detected; -: not determined

Sampling period* (wk)	18 Oct 1990		27 Nov 1990		2 Jul 1991		22 Nov 1991		31 Jan 1992	
	Sulfide	Thiosulfate	Sulfide	Thiosulfate	Sulfide	Thiosulfate	Sulfide	Thiosulfate	Sulfide	Thiosulfate
<i>In situ</i>	342 \pm 217	69 \pm 71 (9)	357 \pm 243	68 \pm 59 (6)	445 \pm 236	21 \pm 22 (10)	353 \pm 232	84 \pm 74 (7)	86 \pm 39	26 \pm 21 (10)
0.5	-	-	-	-	46 \pm 28	14 \pm 4 (4)	-	-	-	-
1	143 \pm 25	nd (5)	172 \pm 71	nd (5)	48 \pm 42	tr (5)	-	-	-	-
2	61 \pm 18	nd (5)	101 \pm 43	nd (5)	-	-	-	-	-	-
3	-	-	-	-	51 \pm 30	tr (5)	-	-	-	-
5	80 \pm 20	nd (5)	-	-	-	-	-	-	-	-
6	-	-	58 \pm 38	nd (3)	-	-	-	-	-	-

* *In situ*: immediately sampled; number indicates weeks after aerated incubation in the lab

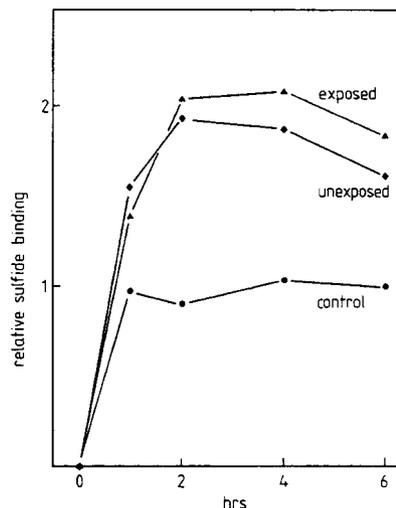


Fig. 4. *Halicryptus spinulosus*. Sulfide binding of buffered seawater (control, 22‰ S) and hemolymph of worms previously unexposed to sulfide and worms exposed for 10 d at 200 μM sulfide prior to binding experiments as function of dialysis duration. Experimental conditions: 9.5 \pm 0.5°C, 22‰ S

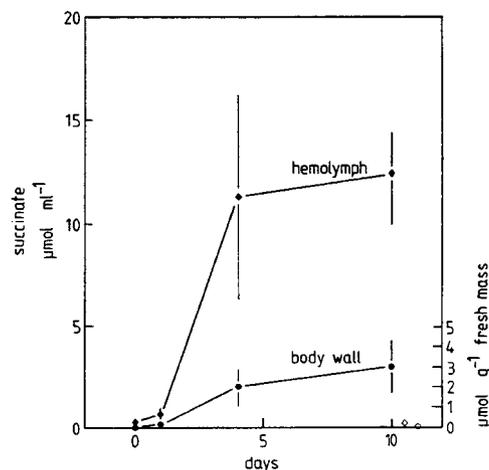


Fig. 5. *Halicryptus spinulosus*. Succinate accumulation \pm SD in body wall tissue (\blacksquare) and hemolymph (\bullet) during oxic sulfide incubation. Measurements are means of 3 to 4 individuals (controls) and 5 to 11 individuals for incubated animals. Single data points (open symbols) after 10 d are control animals kept aerated without sulfide during the incubation experiment. Values are in $\mu\text{mol g}^{-1}$ fresh mass for body wall tissue and $\mu\text{mol ml}^{-1}$ for hemolymph. Experimental conditions: 9.5 \pm 0.5°C, 22‰ S

Activity of cytochrome c oxidase and catalase

Activity of cytochrome c oxidase was low in the hemolymph. *In vitro* inhibition of cytochrome c oxidase by sulfide from body wall tissue (K_i) was 1.05 ± 0.22 μM sulfide, while *in vivo* activity in body wall tissue of individuals exposed to 200 μM sulfide was ca 50 % of that of unexposed controls (Table 2).

In vitro inhibitory constants of catalase for sulfide (K_i) were in the same range for hemolymph (198 ± 25 μM) and body wall tissue (201 ± 32 μM). Activity of catalase in body wall tissue was not altered *in vivo* during 10 d of sulfide incubations (200 μM) (Table 2). Catalase activity in body wall tissue previously sonicated to strip off the mucus covering the worms showed no significant difference to that of untreated worms. Sodium azide, a specific catalase inhibitor, reduced the activity of the enzyme nearly completely. A concentration of 250 μM azide inhibited catalase activity in hemolymph by 86.4 ± 2.8 % ($n=5$), and a concentration of 1 mM inhibited the enzyme 94.4 ± 2.4 % ($n=3$). The activity of the enzyme from body wall tissue was almost entirely inhibited at 250 μM azide (97.8 ± 3.8 %, $n=3$).

Mitochondria

Different isolation procedures were tested. An isolation medium consisting of glycine and sucrose yielded maximal respiratory control rates (RCR). 2-oxoglutarate in the respiratory buffer was a prerequisite to measure respiration rates of *Halicryptus spinulosus* mitochondria. RCRs with succinate (5 mM) as substrate were 1.72 ± 0.12 ($n=11$) with a maximum of 1.9. Mitochondrial preparations were cyanide-sensitive. Sulfide oxidation of the mitochondrial isolation

was fast in the lower sulfide range: up to 11.3 nmol sulfide mg^{-1} protein min^{-1} at 5 μM (i.e. 6.5 nmol sulfide in the assay) and up to 7.06 nmol sulfide mg^{-1} protein min^{-1} at 10 μM (i.e. 13.0 nmol sulfide in the assay). This rate might contain a certain fraction of an unspecific oxidation due to factors other than mitochondria. Sulfide inhibitory levels were 50 μM . More detailed mitochondrial sulfide oxidation characteristics need to be clarified in a further study.

DISCUSSION

When marine animals are confronted with environmental sulfide (its toxicity is attributed to the blocking of cytochrome c oxidase; National Research Council 1979), only limited defence responses are possible. Mobile fauna will be able to escape, unless the affected areas do not exceed small-scale ranges. Adult benthic macrofauna from soft sediments will be unlikely to escape into the water column, since these organisms generally have only very limited swimming capabilities. To live successfully in environments with the risk of frequent or regular exposure to sulfide, benthic macrofauna have to develop sulfide resistance as well as detoxification.

Adaptation to hydrogen sulfide can potentially take many forms in benthic infauna. Some mechanisms have been described but others doubtlessly remain to be elucidated. Mechanisms of sulfide resistance range from simple passive exclusion due to shell closure in bivalves to elaborate mitochondrial detoxification processes that produce ATP from sulfide oxidation (Powell & Somero 1985, O'Brien & Vetter 1990). *Halicryptus spinulosus* possesses some mechanisms known from other invertebrates as well as some new processes.

Aerobic respiration in *Halicryptus spinulosus* does

Table 2. *Halicryptus spinulosus*. Activity of cytochrome c oxidase and catalase in control (no sulfide) and anoxic sulfide exposed (200 μM) worms. Values are means \pm SD in U ml^{-1} for hemolymph or U g^{-1} fresh mass for body wall tissue. Unit definitions: 1 unit of cytochrome c oxidase converts 1 μmol of reduced cytochrome $\text{min}^{-1} \text{ml}^{-1}$ hemolymph or g^{-1} fresh mass of body wall tissue; 1 unit of catalase decomposes 1 μmol of $\text{H}_2\text{O}_2 \text{min}^{-1} \text{ml}^{-1}$ hemolymph or g^{-1} fresh mass of body wall tissue. 50 % inhibitory constants (K_i) are given in $\mu\text{M} \pm$ SD for sulfide. No. of samples are in parentheses. —: not determined

	Cytochrome c oxidase		Catalase	
	Hemolymph (U ml^{-1})	Body wall (U g^{-1} fresh mass)	Hemolymph (U ml^{-1})	Body wall (U g^{-1} fresh mass)
Unexposed controls	0.05 ± 0.02 (5)	0.64 ± 0.2 (10)	102.6 ± 69.1 (5)	454.7 ± 127.5 (14)
Sulfide exposed worms (200 μM) after:				
1 d	—	—	—	462.1 ± 274.9 (6)
8 d	0.03 ± 0.01 (5)	0.37 ± 0.15 (5)	—	—
10 d	—	—	—	343.2 ± 136.7 (12)
K_i for sulfide (μM)	—	1.05 ± 0.22 (5)	198 ± 25 (5)	201 ± 32 (5)

not appear to be resistant to the effects of sulfide. Both intact mitochondria and specific enzymes show sulfide sensitivity at levels comparable to other organisms (Powell & Somero 1986, Bagarinao & Vetter 1990). *H. spinulosus* mitochondria are involved in sulfide oxidation. As in other marine organisms (Powell & Somero 1986, Bagarinao & Vetter 1990, O'Brien & Vetter 1990) mitochondrial oxidation only works when sulfide concentration does not exceed a critical limit. When oxygen is available (as in the respiration chamber), mitochondria will oxidise sulfide. This process is cyanide sensitive. Preliminary studies on *H. spinulosus* mitochondria show that maximal oxidation rates are in the range of 5 to 10 μM sulfide, while inhibitory levels are in the range of 50 μM which is in agreement with other organisms tested so far (compilation in Bagarinao & Vetter 1990). ATP production in marine organisms using the proton potential generated by sulfide oxidation is generally low (Bagarinao & Vetter 1990, O'Brien & Vetter 1990). In the case of *H. spinulosus* the oxidation of sulfide primarily seems to be used as a detoxification process.

In this context it is tempting to speculate about an analogous mechanism known from brown adipose tissue in hibernating mammals and heater cells involved in keeping endothermy in Scombroideid fish. Mitochondria of these tissues are uncoupled from ATP production to solely generate heat (as quoted in Hochachka & Somero 1984, Block 1991). A similar ATP-independent mitochondrial sulfide oxidation clearly would facilitate sulfide detoxification. The involvement of mitochondria in sulfide detoxification in *Halicryptus spinulosus* is also proved by ultrastructural studies: mitochondria of epidermis and body wall tissue show altered electron densities caused by sulfide (Janssen & Oeschger 1992). Moreover there were electron dense inclusions in epidermal mitochondria after sulfide incubation suggesting the deposition of substances resulting from sulfide detoxification.

Halicryptus spinulosus has an *in vitro* sulfide inhibition constant (K_i) of cytochrome c oxidase of ca 1 μM . According to Bagarinao & Vetter (1990) this is in the upper range known for marine organisms. Catalase, another sulfide-sensitive enzyme with a metal-containing catalytic centre (National Research Council 1979), has a much higher *in vitro* inhibition constant, about 200 μM in body wall tissue and the hemolymph. *In vivo* activity of cytochrome c oxidase after 8 d of sulfide incubation (200 μM) is about 50% of oxic control worms, while catalase is not affected (Table 2). Striking differences for cytochrome c oxidase inhibition *in vitro* and *in vivo* are due to different pH conditions in the cuvette (assay carried out at pH 6.0) and during whole worm incubation (pH of ca 8.0 in the seawater). *In vitro* inhibition was tested by adding sulfide directly to the

assayed tissue homogenate of unexposed control worms, while *in vivo* inhibition was determined by measuring actual remaining activity in tissue homogenates after incubation (for a more detailed discussion see Bagarinao & Vetter 1990).

There is some evidence that both enzymes, cytochrome c oxidase and catalase, are mutually connected in their functioning. Morill et al. (1988) report catalase in thiobiotic meiofauna to be sulfide-insensitive like a bacterial pseudocatalase. In *Halicryptus spinulosus* a pseudocatalase is not found, since the enzyme is inhibited by azide, a specific catalase inhibitor. Nevertheless, a high sulfide concentration is needed to inhibit the enzyme *in vitro*, while no inhibition is detected in the range of realistic *in vivo* levels of sulfide (200 μM). Undiminished catalase activity in *H. spinulosus* is against the general acceptance that catalase is important especially in habitats where oxygen and its radicals can reach toxic levels. Catalase might be needed to protect *H. spinulosus* in the presence of sulfide, since inorganic reactions of sulfide can produce oxygen radicals and H_2O_2 (Millero 1986). Inhibition of cytochrome c oxidase by sulfide results in elevated levels of superoxide radicals and H_2O_2 due to increased amounts of reduced forms of respiratory chain components and tissue hypoxia may even aggravate this situation (Khan et al. 1990). In vertebrates hypoxia can activate xanthine oxidase which in the presence of sulfide stimulates the production of O_2^- (cf. Khan et al. 1990). Should sulfide thus produce increased amounts of toxic oxygen compounds, a maintained activity of catalase in the presence of sulfide clearly would be advantageous.

No marine animal is now able to exclude external sulfide from entering into the body (e.g. meiofauna: Powell et al. 1979; Crustacea: Vetter et al. 1987, Vismann 1991b; fish: Bagarinao & Vetter 1989). Under aerobic conditions *Halicryptus spinulosus* is able to keep sulfide concentrations in the body low for a certain time, even at high external sulfide concentrations (200 μM). Under this condition sulfide in body wall tissue increases slightly after 4 d probably due to a decreasing sulfide oxidation capacity. Hemolymphic sulfide concentration only increases somewhat after 10 d. High levels of thiosulfate, a non-toxic detoxification product of sulfide, are found in body wall tissue and hemolymph.

Halicryptus spinulosus is able to keep an internal aerobic sulfide oxidation for ca 4 d. The onset of anaerobic pathways, i.e. sulfide-induced anaerobiosis, is indicated after 1 d due to a slight succinate accumulation, a typical anaerobic metabolite. In the subsequent days the worms accumulate considerable amounts of succinate, indicating an elevated fraction of anaerobiosis. The recourse to anaerobic pathways goes

against the classical definition of anaerobiosis as being triggered by the absence of oxygen. Sulfide as a trigger of anaerobiosis in the presence of oxygen has been reported so far in the case of *Solemya reidi* (Anderson et al. 1990) and most recently in *Sipunculus nudus* and *Arenicola marina* (Völkel & Grieshaber 1992).

When the environment becomes anoxic and sulfidic, *Halicryptus spinulosus* appears to use a short-term defence to control influx of external sulfide. *H. spinulosus* differs from all previously studied organisms in its formation of black, acid-labile iron-sulfide interactions, which may produce Mackinawite-like (FeS) or Greigite-like (Fe₃S₄) compounds, and the ease in which it can apparently reverse this process. The conspicuous blackening of the worms is attributed to sulfide reacting with iron (mostly Fe²⁺) which is present in large amounts on the cuticle. Thus a first protection against sulfide is achieved (Oeschger & Janssen 1991). The effect of this mechanism is confirmed by ultrasonification. When the outer, iron-sulfide containing mucus is removed, body wall tissue contains less sulfide. A similar mechanism is discussed for internal iron deposits supposed to participate in sulfide detoxification in the gut of echinoderms (Buchanan et al. 1980, de Ridder et al. 1985) and in the mid-gut gland of an isopod (Vismann 1991b). An outer shield in *H. spinulosus* seems advantageous when the worms burrow in deeper sediment layers where they may encounter varying concentrations of sulfide (Fig. 1). A certain degree of blackening of the cuticle also occurs during oxic sulfide incubation. About 50% of the incubated worms became black after 3 d. This outer shield is rechargeable as previously reported. Oxygen reverses the colour change (Oeschger & Janssen 1991). The black colour also disappears when treated with acidic solutions.

Such a striking colour change in a marine invertebrate related to sulfide exposure and its involvement in sulfide detoxification has not yet been reported. O₂-independent oxidation of sulfide mediated by iron ions is also reported for anoxic seawater (Millero 1991a, b). At the present we can only speculate about similarities of these mechanisms.

Sulfide oxidation to thiosulfate under anoxia is only of minor importance. At the beginning of the experiments a small increase of thiosulfate in body wall tissue and hemolymph was detected. We attribute this to an oxygen supply limited by the storage capacity of hemerythrin, the respiratory pigment of priapulids. Thus, other mechanisms must be effective. *Halicryptus spinulosus* binds some sulfide to a yet unknown hemolymphic-borne binding factor. Buffering of sulfide by binding should be another short-term defence, although a 2-fold sulfide-concentration capacity in *H. spinulosus* is moderate compared to a 6-fold one in

Escarpia laminata, a vestimentiferan worm (Cary et al. 1989a). Sulfide binding proteins have so far only been found in marine animals with endosymbionts (Arp et al. 1984, 1987, Cary et al. 1989a) and might also be present as an iron-protein complex in the mid-gut gland of *Saduria entomon* (Vismann 1991b). The hemolymphic binding factor of *H. spinulosus* explains a 2- to 3-fold accumulation of sulfide over external levels, which would be impossible according to the laws of diffusion. High hemolymphic sulfide levels (up to 80 μM) after 5 to 6 wk of aerated incubation (Table 1) or in controls (Fig. 3a) indicate that the binding factor releases sulfide very slowly. This is supported by the absence of thiosulfate after prolonged incubation (Table 1). Sulfide binding in *H. spinulosus* may result in a certain fraction of acid-volatile sulfide as reported for *Saduria entomon* (Vismann 1991b). This still has to be elucidated for the priapulid worm. In such a case elevated sulfide levels are attributed to a bound acid-labile fraction which does not harm the animals.

A distinct colour change of the hemolymph of *Halicryptus spinulosus* after ca 3 d of anoxic sulfide incubation indicates that the hemerythrin reacts with sulfide. Hemerythrin is known to contain a substantial amount of iron (Klotz et al. 1957), so that an iron-sulfide interaction seems likely. In contrast to hemoglobin, hemerythrin binds oxygen by changing its valence from Fe²⁺ to Fe³⁺ (Klotz et al. 1957, Klippenstein 1980). Our preliminary studies on the coelomic fluid show that the black component is associated in the erythrocytes and has a molecular weight > 10 kDa, when we used a centrifugation procedure that excludes molecules of this size. Thus we can rule out formation of a free iron-sulfide complex, which would have passed the membrane due to its molecular size. At this time we can only speculate on a binding to the hemerythrin since hemolymph regains its usual pinkish colour when oxygen is present. Our findings are in accordance with Schreiber et al. (1990), who reported a major hemolymph iron-containing constituent of ca 12 kDa with tightly bound iron. This binding is not disturbed by treatments of strong detergents, reducing agents or heat denaturation, and represents the respiratory pigment, i.e. hemerythrin, of the animals. Sulfide binding implies a release at a later time. Presently we do not know if the sulfide will be released when the pigment is reoxidised or if it is released as an oxidised form of sulfide. Presumably the oxygen-binding capacity will be destroyed by sulfide binding. It has yet to be elucidated if the sulfide binding means a beneficial adaptation or if it is a mere chemical interaction between sulfide and respiratory pigment.

Binding of sulfide may at least temporarily prevent mitochondria in *Halicryptus spinulosus* from being poisoned since these organelles are obviously important

in sulfide detoxification. But this may only work as a short-term protection depending on the oxygen storage capacity of the hemerythrin. A putative enzymatic sulfide oxidase activity separate from mitochondria has previously been reported (Powell & Somero 1985). Due to non-physiological assay conditions the method used to demonstrate such a specific enzymatic activity has been doubted (Bagarinao & Vetter 1990). So far there is no evidence for a specific enzyme and its physiological relevance. Spontaneous sulfide oxidation catalysed by metal ions (Chen & Morris 1972) may be a large portion of the 'sulfide oxidase' activities. Thus mitochondrial sulfide oxidation confirmed for the bivalve *Solemya reidi* (Powell & Somero 1986) and marine fish (Bagarinao & Vetter 1990) might be the only well-documented sulfide oxidation system at physiologically realistic conditions.

This study also suggests that anaerobiosis is involved in sulfide resistance. *Halicryptus spinulosus* has a remarkable anaerobic capacity (Oeschger 1990). Succinate accumulation during oxic sulfide incubation shows that the worms rely to an increasing degree on anaerobic metabolism. Obviously *H. spinulosus* has to switch to anaerobiosis when sulfide influx becomes too high for detoxification to keep up. Worms will be forced to passively survive this adverse environmental condition by becoming metabolically quiescent. Calorimetric studies prove an enormous metabolic reduction (Oeschger et al. 1992). Such a reduction will also be an advantage during elevated sulfide exposure. In *H. spinulosus* anaerobic glycolysis is not affected by the presence of sulfide over 10 d (Oeschger & Storey 1990). On the other hand one has to take into consideration that accumulating acidic anaerobic metabolites will cause a substantial drop in internal pH. Such a decrease intensifies sulfide toxicity. Thus the accumulation of acidic end-products might be an essential factor affecting long-term survival. At a physiological pH of ca 7.6 to 8.0 the toxic form of sulfide, the uncharged molecule H_2S , is present at 10 to 20% (APHA 1985). When pH drops, e.g. to 7.0, ca 50% of sulfide will be present in the uncharged form (H_2S) which will readily penetrate membranes. Even though glycolysis is not affected in *H. spinulosus*, an aggravation due to elevated sulfide influx with concomitant increase of undissociated toxic H_2S as a result of a decreasing internal pH seems to be unavoidable. During long-term sulfide exposure this may lead to fatal interferences with metabolic processes.

Whole animal model of sulfide control

The presented data allow an overall model of sulfide detoxification and resistance to be made in a marine

invertebrate from soft sediment. External sulfide is partly immobilised on the outer mucus barrier by precipitating iron-sulfides, made visible by blackening of *Halicryptus spinulosus*. When sulfide exposure prevails above the precipitation capacity, toxic sulfide entering the worm is oxidised to thiosulfate, the major detoxification product. Thiosulfate production depends on oxygen availability. When sulfide concentration exceeds a specific threshold, aerobic pathways are supplemented by an increasing fraction of fermentative ATP production.

Simultaneously or upon exhaustion of oxygen or detoxification capacity in mitochondria, a hemolympic binding factor, which seems identical with hemerythrin, also limits free sulfide concentration. This process may help to keep mitochondria from being poisoned to a certain degree. Later the hemerythrin also blackens when obviously being involved in sulfide binding. When these mechanisms of sulfide control are overwhelmed by further sulfide influx, *Halicryptus spinulosus* switches entirely to anaerobic energy metabolism. This is to passively outlast sulfide. On return to oxic non-sulfidic conditions, worms regain their pale colour by reversible iron-sulfide splitting. *H. spinulosus* can reverse inorganic iron-sulfide formation and restore hemerythrin function. Further research should be directed to elucidating the nature of these protein-bound, iron-sulfur compounds and the biochemical mechanism of their reversal.

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