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Sulphide tolerance of the marine nematode *Oncholaimus campylocercoides*— a result of internal sulphur formation?

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ABSTRACT: The free-living, marine nematode *Oncholaimus campylocercoides* occurs in high abundance (up to 600 ind. 10 cm^{-2}) at the fringe area of shallow-water hydrothermal vents off the Greek island of Milos in the Aegean Sea. It was found to have a sulphide tolerance (LT_{50}) of 4.5 d at 500 µM sulphide concentration and of 4 d at 1 mM sulphide. Light- and electron-microscopical inspections showed that the non-symbiotic *O. campylocercoides*, when exposed to sulphidic conditions, develops oily to viscous inclusions in the epidermis consisting of elemental sulphur in the form of S_8-rings and polysulphur chains. The longer the exposure to sulphidic conditions, the more sulphur was formed, which disappeared after re-introduction of the nematodes in normoxic conditions for 12 h. Based on these results and on tolerance experiments with hydrogen sulphide, we suggest a model of sulphide metabolism in *O. campylocercoides* which could relate to its occurrence in sulphidic, hydrothermal sediments.

KEY WORDS: Hydrothermal vents · Nematodes · Oncholaimidae · Sulphur inclusions · Sulphur metabolism · Thiobios · Hydrogen sulphide

INTRODUCTION

The free-living marine nematode *Oncholaimus campylocercoides* (Oncholaimidae, Enoplida) dominates the fauna of sediments around shallow-water hydrothermal vents off the Greek island of Milos (Thiermann et al. 1994, 1997). Despite sulphide concentrations of 20 to 300 µM, pH values of 6.5 to 7, elevated temperatures (25 to 30°C) and salinities ranging from 39 to 46 ppt, the species occurs in abundances up to 600 ind. 10 cm^{-2}.

Apparently surviving the geothermal sulphide concentrations of Paleohori Bay better than most other benthic species (Thiermann et al. 1994), the predator and omnivorous scavenger *Oncholaimus campylocercoides* (Teal & Wieser 1966, Jensen 1987) might benefit from the ample supply of moribund or dead worms, poisoned by hydrogen sulphide, in a habitat with low competition.

Oncholaimus campylocercoides, however, is not restricted to sulphidic areas. The species is regularly found in shallow sediments of the Mediterranean and boreal seas, occurs also in brackish water (Baltic Sea, Black Sea), and tolerates even the hypersaline conditions in the Egyptian part of the Red Sea (Gerlach & Riemann 1973).

Earlier investigations of *O. campylocercoides* (Thiermann et al. 1994) showed that this species developed sulphur-containing droplets when exposed to hydrogen sulphide. In this paper, data from ecological experiments, structural and physiological investigations are combined to interpret the sulphide tolerance of *O. campylocercoides* for hydrogen sulphide, and to relate these results to its field distribution in a hydrothermal gradient system.

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MATERIAL AND METHODS

Cultivation of nematodes. Specimens of *Oncholaimus campylocercoides* were sampled at shallow-water gaseohydrothermal vents off the Greek island of Milos (see Thiermann et al. 1994, 1997). The worms were extracted and cultivated in aquarium tanks (10 × 15 cm) containing a 3 to 5 cm layer of natural, but azoic (by freezing twice) sediment in aerated seawater (salinity 35 ppt) at room temperature. The populations were fed with commercial fish food flakes (Tetramin®). When co-cultured with *Capitella capitata* (Polychaeta), the populations could be kept for several years and reproduced in high numbers.

Tolerance experiments. The mean survival rates of *Oncholaimus campylocercoides* (% survivors) were studied under 4 experimental conditions. In each experiment 25 specimens were incubated at room temperature in glass jars (vol = 60 ml) with artificial seawater (salinity = 35 ppt). Initially pH was adjusted to pH 7.5 (HCl, NaOH, HEPES buffer) and measured again at the end of the experiment. Severe hypoxia was obtained by percolation with nitrogen for 1 h, which reduced the oxygen tension below the detection limits of polarographic oxygen electrodes (<1 µmol O_2 l^{-1}, e.g. Gamenick et al. 1998a). The appropiate sulphide concentrations were achieved by addition of a 10 mM solution. Sulphide concentrations were measured colourimetrically after Howarth et al. (1983) at the start and the end of the experiments (Table 1).

After insertion of the nematodes, the jars were closed and submerged in a seawater bath which was permanently percolated with nitrogen to prevent influx of oxygen. Normoxic control experiments were performed in a water bath that was aerated with atmospheric air. Here, the jars remained open and in direct atmospheric contact. Survival of the nematodes was recorded every second hour under a dissection microscope without removing the worms from the experimental jars. Nematodes were considered dead when no tactile response was observed after stirring. Hence, 'mortality' in the present paper refers to both anaesthetized and dead worms.

Light microscopy. For analytic inspection of sulphur inclusions, specimens of *Oncholaimus campylocercoides* were incubated under hypoxic (<1 µmol O_2 l^{-1}) and sulphidic conditions (100 ± 7.3 µM) for 30 min, 1, 2, 4 and 8 h (pH = 7.0, salinity = 35 ppt) in 50 ml glass containers (Table 1). The glass containers were closed with plastic lids and submerged in hypoxic water (continuous N_2-bubble stream as described above). A control group was incubated in fully aerated water (pH = 7.5, salinity = 35 ppt). At the end of the incubation period, the worms were fixed with formalin (5%) and mounted according to conventional methods (see Riemann 1988). For light microscopy a compound microscope (Zeiss Standard) with interference contrast was used.

Transmission electron microscopy. Specimens of *Oncholaimus campylocercoides* were incubated as described for the light microscopical studies. Immediately after the end of the incubations, the worms were fixed in Trump's fixative (McDowell 1978) buffered in sodium cacodylate. Specimens were washed in cacodylate, postfixed in 1% osmium tetroxide, dehydrated in an acetone series (up to 70%) and embedded in Spurr's resin. Ultrathin sections, mounted on copper grids and contrasted in aqueous uranyl acetate and lead citrate, were examined in a transmission electron microscope (Zeiss EM 902 A). Uncontrasted ultrathin sections were used to perform element analysis by Electron Energy Loss Spectrography (EELS and ESI) linked to the Zeiss electron microscope. Despite the considerable solubility of sulphur in acetone, this method allowed exact location of chemical elements in ultrathin sections of the tissues (Simon 1988).

Scanning electron microscopy (SEM). For scanning electron analysis, specimens of *Oncholaimus campylocercoides* were fixed in Trump's fixative and rinsed with cacodylate buffer. Subsequently the worms were dehydrated in a gradient series of acetone and critical point dried. The worms were then torn open longitudinally by rolling them carefully over the adhesive film of the mounting plate with a fine brush and finally gold sputtered. For inspection a Cambridge Camscan DV 4 was used.

Analysis of sulphur metabolism. All exposure experiments were done under constant pH, sulphide, oxygen and temperature conditions which were automatically controlled, monitored and adjusted by a computer (see Vismann 1996, for details of the set-up). The salinity was set at 35 ppt (seawater salt) and controlled with a hand-refractometer (Atago).

Batches of 150 nematodes each were transferred into loop-shaped pvc-hoses (5 mm i.d.)

Table 1. H_2S and pH values of the tolerance experiments (µM = µmol l^{-1}). (A) nominal sulphide concentration: 1 mM and (B) nominal sulphide concentration: 0.5 mM

Replicate	H_2S/pH (start)	H_2S/pH (end)	mean H_2S/pH
(A) 1 mM			
1	970 µM/pH 7.5	792 µM/pH 7.43	881 µM/pH 7.46
2	982 µM/pH 7.5	837 µM/pH 7.45	909 µM/pH 7.47
3	976 µM/pH 7.5	874 µM/pH 7.48	925 µM/pH 7.49
(B) 0.5 mM			
1	640 µM/pH 7.5	472 µM/pH 7.03	556 µM/pH 7.2
2	631 µM/pH 7.5	492 µM/pH 7.31	561 µM/pH 7.45
3	627 µM/pH 7.5	478 µM/pH 7.27	552 µM/pH 7.39

containing natural sediment. In order to expose the worms to exactly comparable conditions, the sediment-filled loops were supplied with water from the same water tank by a peristaltic pump. Three replicate experiments were performed for each experimental run.

In this paper the term hypoxia is used for oxygen concentrations below 10% atmospheric saturation. Even when oxygen concentrations were below the detection limits of O_2-electrodes, we considered the conditions to be hypoxic, not anoxic.

Expt 1: exposure periods of increasing length. Specimens of *Oncholaimus campylocercoides* were exposed to constant hypoxia (10% oxygen saturation) and sulphide (100 µM) for 0.5, 1, 1.5, 2, 4 and 8 h. pH was 7.5 and temperature about 14°C (Table 2).

Expt 2: normoxic conditions of varying lengths after sulphide exposure. After 4 h exposure to 100 µM sulphide, the worms were put into fully aerated, non-sulphidic sediment for 3 and 12 h.

Chemical analysis of sulphur species. Immediately after experimental incubation (see above), the worms were fixed in formalin (5%) and washed in pure chloroform to remove any traces of sulphur from the body surface. They were then ultrasonically homogenised in chloroform in ice-cold test tubes and the chloroform was allowed to completely evaporate. By addition of 200 µl chloroform all the internal sulphur was dissolved during a period of 36 h.

Elemental sulphur was analysed by high-performance liquid chromatography (HPLC) using a pump (Jasco 880 PU), a manual injector (Rheodyne 7105) fitted with a 20 µl loop, and a UV/VIS detector (Jasco 875-UV) set at 254 nm. Treatment of the column (Hamilton PRP reversed phase, 15 cm × 4.1 mm i.d.) prior to analysis, extraction of samples in chloroform, subsequent uptake in methanol, and the HPLC protocol were performed according to Lauren & Watkinson (1985).

Table 2. Expt 1. Experimental conditions of the computer-controlled incubations for sulfur determination (µM = µmol l^{-1})

Exposure time (h)	Concentration O_2 (%)	H_2S (µM)	pH	Temperature (°C)
0.5	9.84 ± 0.66	97.1 ± 8.2	7.502 ± 0.003	14.6
1.0	9.24 ± 0.78	91.0 ± 7.3	7.508 ± 0.006	14.4
1.5	9.57 ± 0.81	96.5 ± 8.5	7.502 ± 0.002	13.7
2.0	9.74 ± 0.72	92.5 ± 7.5	7.506 ± 0.005	13.7
4.0	10.08 ± 1.1	93.7 ± 8.7	7.511 ± 0.01	14.7
8.0	9.73 ± 0.76	93.6 ± 6.2	7.527 ± 0.016	13.7

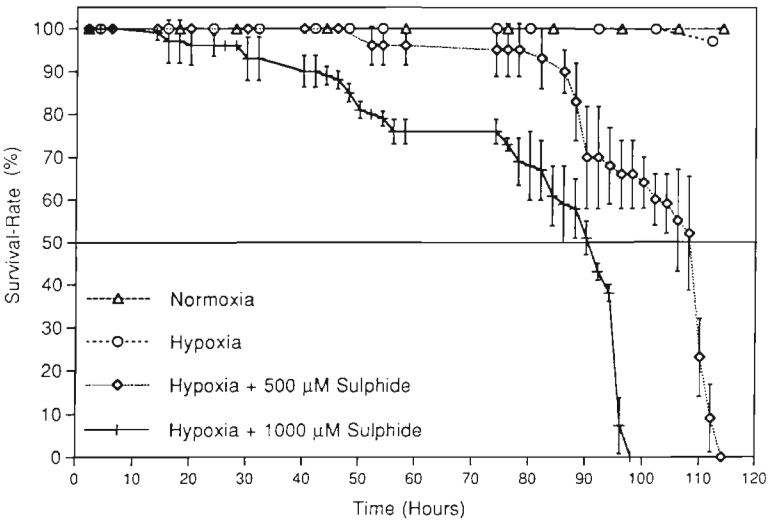

Fig. 1. *Oncholaimus campylocercoides*. Mean survival rates (%) and standard deviations when exposed to normoxia, hypoxia and hypoxia plus 905 (1000) and 556 (500) µmol l^{-1} sulphide

Statistical analysis. The data are presented as mean values with their standard deviation. For all data, the non-parametric Mann-Whitney *U*-test was used at the 0.05 level to test if differences were significant.

RESULTS

Sulphide tolerance

Hypoxic conditions did not significantly reduce the survival of *Oncholaimus campylocercoides* within time periods up to 115 h (Fig. 1). Only 1 worm died after 110 h. Under sulphidic conditions (500 µM), worms began to die after 84 h, the mean survival rate (LT_{50}) was about 108 h, and 100% mortality was recorded after 114 h.

After the sulphide concentration was doubled (1000 µM), the onset of mortality was reduced to 32 h exposure time while the mean survival rate (LT_{50}) was 94 h. After 99 h, none of the worms reacted to tactile stimuli. In contrast, in the control group, all worms survived the experiment.

Structure

Worms exposed to sulphidic conditions (400 µM), regardless of their exposure time, developed droplet-shaped, oily inclusions in their epidermal tissue (Fig. 2a). The oily content exuded in squeeze preparations. These droplets were soluble in ethanol, but not in water. In none of the control animals (normoxic conditions) were similar structures observed (Fig. 2b).

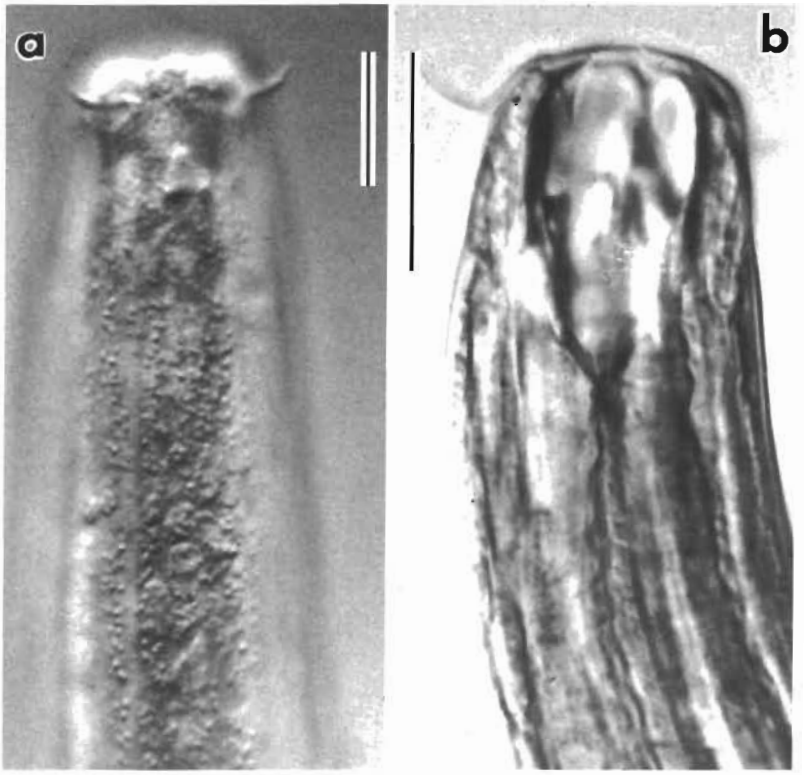

Fig. 2. *Oncholaimus campylocercoides*. Light micrographs (a) after exposure to sulphide (100 µmol l^{-1}, 30 min). (note epidermal inclusions) and (b) after 12 h exposure to normoxic, non-sulphidic conditions. Scale bars = 20 µm

The oily droplets corresponded in size and position to electron-lucent, amorphous and non-menbrane-bound inclusions in the epidermal layer (1 to 3 µm in diameter) when inspected in the electron microscope (Figs. 3 & 4). EELS and ESI spectra proved that these inclusions contained high concentrations of sulphur (Figs. 5 & 6).

Ecophysiology

All sulphide-incubated specimens of *Oncholaimus campylocercoides* were found to contain a considerable amount of elemental sulphur (S^0) which occurred in the form of S_8-rings and polysulphur chains (-S-S_n-S-).

Already after 0.5 h of exposure, the S_8 concentration within the worms was 170 µg g^{-1} ww. Longer incubation times did not lead to significantly higher S_8 concentrations (180 µg g^{-1} ww after 8 h incubation time) in the tissues of *Oncholaimus campylocercoides*. In contrast, polysulphur concentrations increased significantly with exposure time (Fig. 7). Polysulphide concentrations are interpreted here as peak areas (height and width of peaks correlate with concentration). Quantification of polysulphur in absolute terms of S concentration was not possible, as polysulphur standard solutions were not available.

After 12 h of exposure to normoxic conditions, the sulphur (S_8 and polysulphides) that had accumulated during sulphide exposure had disappeared. After 3 h, however, a significant decrease of sulphur could not yet be observed (Fig. 8).

DISCUSSION

Sulphide tolerance

Published information on the tolerance of free-living marine nematodes for controlled hypoxic/sulphidic conditions does, as yet, not exist. There is an unpublished record (Stuhlmacher & Jensen) on the surface-dwelling species *Sigmophoranema rufum*, which immediately died after experimental exposure to 100 µM sulphide. Some data are available on survival times of nematodes under normoxic and hypoxic conditions: In *Theristus anoxybioticus* (Jensen 1995) the adults were less resistant

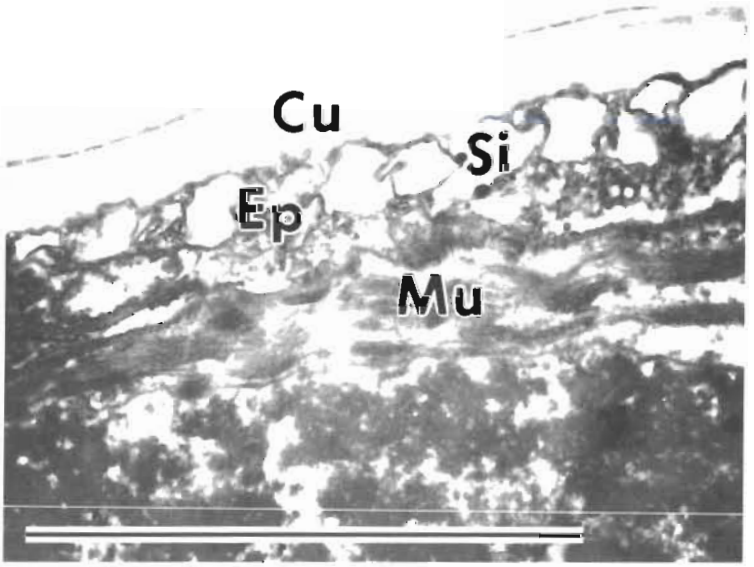

Fig. 3. *Oncholaimus campylocercoides* after sulphidic incubation (100 µmol l^{-1}, 30 min), cross-section through the epidermis and cuticle. Note electron-lucent inclusions in the epidermal layer. Cu: cuticle; Ep: epidermal layer; Mu: muscle-cells; Si: sulphur inclusion. TEM micrograph, scale bar = 5 µm

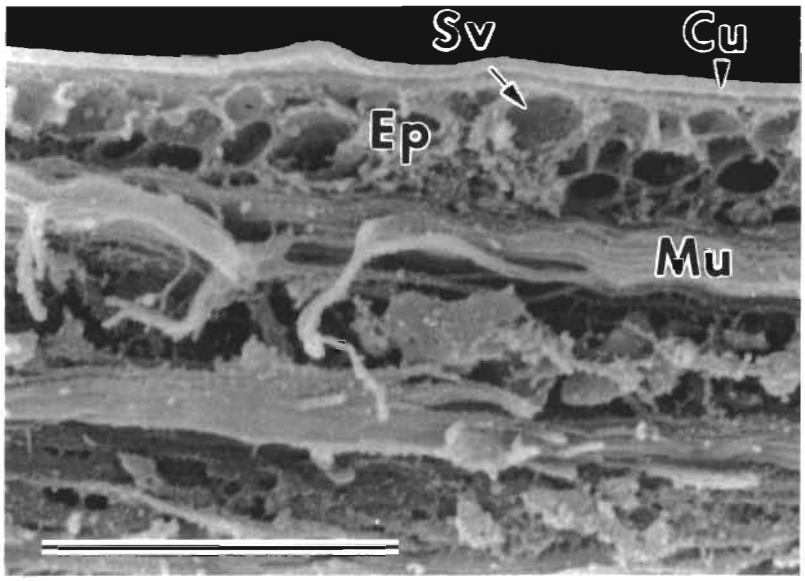

Fig. 4. *Oncholaimus campylocercoides* after sulphidic incubation (100 µmol l^{-1}, 30 min). Note vacuoles of sulphur inclusions in the epidermal layer. Cu: cuticle; Ep: epidermal layer; Mu: muscle-cells; Sv: sulphur vacuole. SEM micrograph, scale bar = 5 µm

than the juveniles. The limnic species *Eudorylaimus andrassyi* from the anaerobic benthal of Lake Tiberias showed experimental resistance of up to 8 mo to anoxic/sulphidic conditions (Por & Masry 1968). Since in that experiment oxygen or sulphide concentrations have not been recorded, a comparison to the data on *Oncholaimus campylocercoides* is not possible. Also various scattered reports on freshwater and marine nematodes surviving long periods under completely anoxic/sulphidic natural conditions need experimental scrutiny for detailed comparison. The survival time of 4 d under highly sulphidic conditions, shown here for *O. campylocercoides*, is in accordance with the co-occurring polychaete *Capitella capitata* sp. M (sensu Gamenick et al. 1998a), which survived comparable sulphidic conditions for 4.4 d (Gamenick et al. 1998b). Longer survival rates (LT_{50} = 27 d under highly sulphidic conditions [1.8 mM]) of a meiobenthic organism has as yet only been shown for the ostracod *Cyprideis torosa* (Gamenick et al. 1996).

Sulphide metabolism

The significance of sulphur formation in metazoans cannot be adequately addressed without discussing—in close context—the metabolic pathways and detoxification effects of hydrogen sulphide oxidation. The main toxic effect of hydrogen sulphide is the reversible blocking of the cytochrome-c-oxidase, an important enzyme of the oxidative phosphorylation (e.g. National Research Council 1979, Vismann 1991, Bagarinao 1992). Thus, from a chemical point of view, any oxidative pathway leading to non-toxic sulphur compounds such as thiosulphate, sulphate, metal sulphides or elemental sulphur can be formally regarded as a process of detoxification. However, this interpretation does not adequately consider the physical or physiological processes involved. When Powell (1989) calculated the diffusion rate of hydrogen sulphide into animals, he came to the conclusion that any detoxification mechanism would be too slow to effectively protect small invertebrates such as meiobenthos from the influx of hydrogen sulphide. In consequence, he contended that meiofauna from sulphidic habitats must possess a sulphide insensitive respiration chain for continuous mitochondrial energy production.

Even in larger animals, an effective protection, e.g. by precipitation of sulphide as insoluble metal sulphides, has been refuted on the basis of careful mathematical and topological evaluations (Dubilier et al. 1994, Jahn et al. 1996, 1997). By theoretical deduction and experimental scrutiny, Jahn et al. (1997) confirmed that animals smaller than 1 mm are 'not able to detoxify hydrogen sulphide effectively enough but rather can only survive in sulphidic habitats due to their anaerobic capacity'. Most nematodes are longer than 1 mm, but their small diameter allows H_2S to immediately enter the whole body by diffusion.

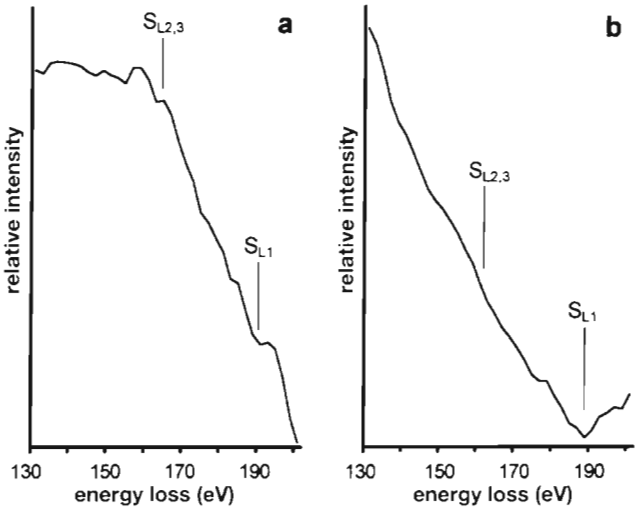

Fig. 5. *Oncholaimus campylocercoides* after sulphidic incubation (100 µmol l^{-1}, 30 min), epidermis cells: (a) EELS-spectra of electron-lucent inclusions and (b) of the adjacent cytoplasm

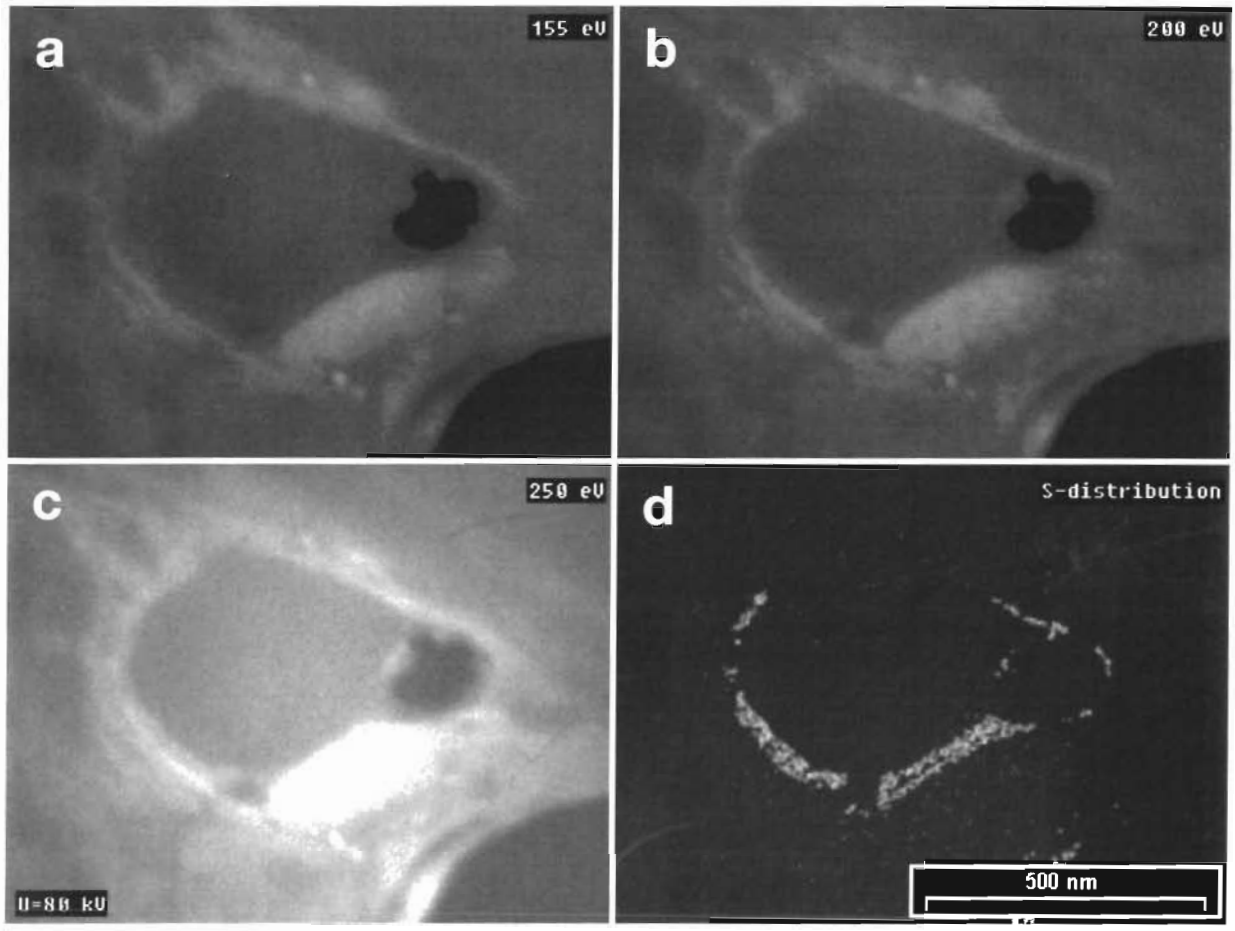

Fig. 6. *Oncholaimus campylocercoides* after sulphidic incubation (100 µmol l^{-1}, 30 min), epidermis cells: ESI-micrograph of electron-lucent inclusion. (a,b) Electron-energy loss values, (c) high-contrast image, and (d) sulphur distribution

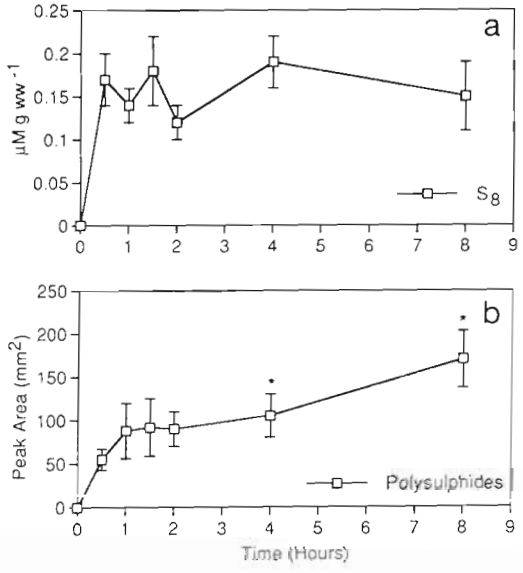

Fig. 7. Sulphur concentrations in *Oncholaimus campylocercoides*, exposed to sulphidic conditions, as a function of time. (a) S_8, and (b) polysulphides (mean H_2S concentration 94 µmol l^{-1}). *Significant difference to the 0.5 h value

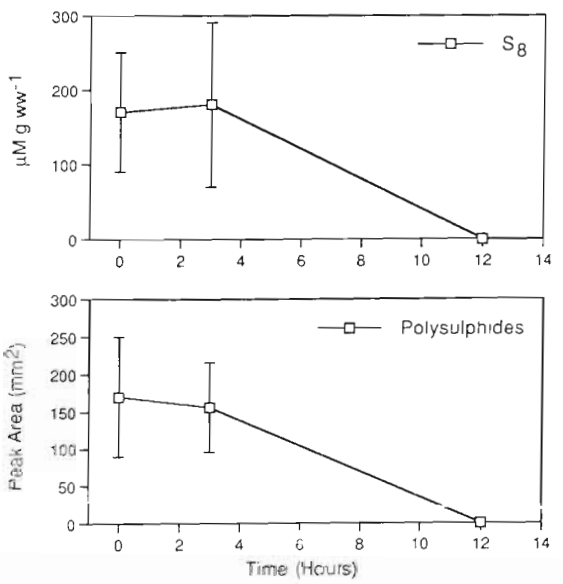

Fig. 8. Sulphur concentrations in *Oncholaimus campylocercoides* when exposed to normoxic, non-sulphidic conditions after 4 h of sulphidic incubation (400 µmol l^{-1})

Ecophysiologically, a sulphide detoxification mechanism or an insensitive respiration chain is of advantage only in a temporarily anoxic/sulphidic environment. Here, maintenance of an oxidative metabolism for a short while would be sufficient to bridge the time until the next chance for a supply of oxygen occurs. In contrast, long-term exposure to fully anoxic and sulphidic conditions will always force the worms to switch to non-oxidative metabolism which, in turn, renders insensitivity to sulphide irrelevant. This mechanism has been shown for the ostracod *Cyprideis torosa* (Jahn et al. 1996), which survived sulphidic conditions for 27 d (Gamenick et al. 1996).

Most members of the thiobios experience only temporary anoxia and exposure to hydrogen sulphide. Their preferred environment is the complicated 3-dimensional network of oxic/sulphidic microniches with interfaces often of microscopical scale (Meyers et al. 1987, 1988, Watling 1991, Giere 1993, Fenchel 1996). In this habitat of frequent but short-term exposure to sulphide any mechanism extending the functioning of the (aerobic) respiration chain, be it by development of sulphide insensitivity, or by (temporary) reduction of the internal sulphide concentration through pathways of sulphide oxidation, would be of energetic benefit.

Energy production by sulphide oxidation, as postulated by Powell (1989) for thiobiotic meiofauna, can only be achieved if hydrogen sulphide entering the body is used for ATP production. Mitochondrial oxidation of H_2S with concomitant ATP production has been shown in the polychaetes *Arenicola marina* (Völkel & Grieshaber 1997) and *Heteromastus filiformis* (Oeschger & Vismann 1994), in some marine fish (Bagarinao & Vetter 1989) and mussels (Powell & Somero 1986). It remains to be proven also in the minute nematode *Oncholaimus campylocercoides.*

In fact, for meiobenthic fauna, Powell's (1989) contention of a sulphide insensitive respiration chain has not been documented (Bagarinao 1992). Our tolerance experiments proved that *Oncholaimus campylocercoides* is not sulphide insensitive. Although it tolerated relatively high sulphide concentrations (up to 500 and 1000 µM), the limited survival periods point to a detrimental or even toxic effect of H_2S.

Formation of sulphur inclusions

Sulphide oxidation forming sulphur inclusions has been discussed as an option to reduce the risks of sulphide poisoning. As reported by Thiermann et al. (1994) sulphur-containing inclusions developed in a reproducible manner in the epidermal tissue of *Oncholaimus campylocercoides* when exposed to hypoxic/sulphidic conditions The amount of elemental sulphur stored as epidermal inclusions correlated well with the time of exposure to ambient sulphide. Accumulation of elemental sulphur in the form of oily to viscous inclusions consisting of S_8 and polysulphur chains is well known from sulphur-oxidising bacteria (Steudel 1989, Jannasch 1995). This phenomenon has also been reported from marine 'sulphur bacteria' living in symbiosis with invertebrate metazoans (review by Fisher 1990).

Since in *Oncholaimus campylocercoides* microscopic examination did not reveal symbiotic bacteria, neither on the cuticle, nor in the nematode's tissues, the elemental sulphur found must be derived from the worm's own metabolism. This reversible and reproducible process of sulphur formation seems a rare metabolic pathway in non-symbiotic metazoa and is experimentally demonstrated in this study for the first time.

Based on the results presented, we suggest the following 'model' of sulphide metabolism in this nematode: (1) Under hypoxic, sulphidic conditions, H_2S enters the body by diffusion. (2) As long as there is oxygen available in the tissues, H_2S will become chemically oxidised to S_8 and polysulphur chains and temporarily stored in the form of oily inclusions in the epidermis, from where it can be easily mobilized. (3) With the re-entry of oxic conditions, the elemental sulphur is oxidized to a water-soluble form (thiosulphate or sulphate) which can be eliminated from the worm's body.

The sipunculid *Phascolosoma arcuatum* seems to possess a comparable process of elemental sulphur formation. Sulphur chains (sulphane-sulphur) have been detected in the body wall and introvert of this species that lives regularly exposed to sulphide in the upper eulittoral of mangrove flats (Ip et al. 1997). Among thiobiotic meiofauna, refractile (crystalline) inclusions containing sulphur have been found in different taxa, albeit without subsequent experimental scrutiny: *Tobrilus gracilis* (Nematoda) (Nuß 1984, Nuß & Trimkowski 1984), *Peudohaplogonaria* sp. (Turbellaria) and *Dolichodasys carolinensis* (Gastrotricha) (Powell et al. 1980).

Sulphur peaks (often together with metals), recorded in granules or intracellular inclusions in various non-bacteria-symbiotic meiobenthic and macrobenthic fauna, have led to the suggestion that elemental sulphur or insoluble metal sulphides, accumulating in the body, are the results of H_2S detoxification (Powell et al. 1980, Nicholas et al. 1987, Giere et al. 1988). However, these and other studies could not convincingly document the effective export mechanisms required to remove the inclusions from the tissues (Somero et al. 1989). Moreover, most reports proposing detoxification mechanisms in 'sulphide animals' by

non-bacterial formation of 'sulphur inclusions' (e.g. Nuß 1984, Nuß & Trimkowski 1984, recently Maina & Maloiy 1998, Menon & Arp 1998), do not satisfactorily consider the previously discussed body-size-related problems of diffusion/detoxification equilibrium and the required influx/efflux balance of sulphur compounds (Powell 1989, Jahn et al. 1997).

Moreover, it is rarely considered that any metabolic pathway removing hydrogen sulphide by oxidation will enhance the diffusive gradient and, thus, the influx of hydrogen sulphide (Powell 1989). This increased influx would counteract any attempt to reduce the toxic impact.

For *Oncholaimus campylocercoides*, living in oxic/sulphidic transition zones, the oxidation of hydrogen sulphide to elemental sulphur could be of advantage for the following reasons: (1) the formation of elemental sulphur temporarily reduces the concentration and toxic effect of H_2S; and (2) the accumulation of elemental sulphur provides an energetic 'deposit' for later oxidation to thiosulphate, sulphite or sulphate under oxic conditions.

This mechanism, shown in the present study, would parallel the oxidation of sulphide in 'sulphur-bacteria' such as *Beggiatoa*. The corresponding formation of oily, metabolically rapidly activated sulphur droplets (Steudel 1989) corroborates this conclusion.

In conclusion, we infer that sulphide detoxification via formation of elemental sulphur has to be considered a pathway effective only in conjunction with other metabolic strategies (review by Grieshaber & Völkel 1998). As a synergistic suite, these adaptive pathways allow the animals to tolerate sulphidic conditions and to benefit from the 'sulphide niche' Taken *per se*, formation of elemental sulphur inclusions can only render a limited and temporary protection against hydrogen sulphide.

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