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The hydrocarbon seep tubeworm *Lamellibrachia luymesi* primarily eliminates sulfate and hydrogen ions across its roots to conserve energy and ensure sulfide supply

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Summary

***Lamellibrachia luymesi* (Polychaeta, Siboglinidae) is a deep-sea vestimentiferan tubeworm that forms large bush-like aggregations at hydrocarbon seeps in the Gulf of Mexico. Like all vestimentiferans, *L. luymesi* obtains its nutrition from sulfide-oxidizing endosymbiotic bacteria, which it houses in an internal organ called the trophosome. This tubeworm has a lifespan of over 170 years and its survival is contingent upon the availability of sulfide during this long period. In sediments underlying *L. luymesi* aggregations, microbes produce sulfide by coupling sulfate reduction with hydrocarbon oxidation. *L. luymesi* acquires sulfide from the sediment using a root-like posterior extension of its body that is buried in the sediment. Its symbionts then oxidize the sulfide to produce energy for carbon fixation, and release sulfate and hydrogen ions as byproducts. It is critical for the tubeworm to eliminate these waste ions, and it could do so either across its vascular plume or across its root. In this study, we measured sulfate and proton elimination rates from live *L. luymesi* and found that they eliminated approximately 85% of the sulfate produced by sulfide oxidation, and approximately 67% of the protons produced by various metabolic processes, across their roots. On the basis of experiments using membrane transport inhibitors, we suggest that *L. luymesi* has anion exchangers that mediate sulfate elimination coupled with bicarbonate uptake. Roots could be the ideal exchange surface for eliminating sulfate and hydrogen ions for two reasons. First, these ions might be eliminated across the root epithelium using facilitated diffusion, which is energetically economical. Second, sulfate and hydrogen ions are substrates for bacterial sulfate reduction, and supplying these ions into the sediment might help ensure a sustained sulfide supply for *L. luymesi* over its entire lifespan.**

Key words: *Lamellibrachia luymesi*, hydrocarbon seep, tubeworm, root, sulfate, proton.

Introduction

Elimination of metabolic waste products is vital for all living organisms. Excess nitrogen, carbon dioxide and water are the major metabolic wastes of most invertebrates (Brusca and Brusca, 2002). However, obligate symbioses, including vestimentiferans, have the additional burden of eliminating waste products generated by their symbionts. Vestimentiferan tubeworms lack a mouth or gut, and derive their nutrition from intracellular endosymbiotic bacteria housed in an organ called the trophosome (Cavanaugh et al., 1981; Jones, 1981). These bacteria oxidize hydrogen sulfide and produce sulfate and hydrogen ions as byproducts, and the tubeworm's mechanism for eliminating these wastes is among its suite of adaptations crucial for this symbiosis (Girguis et al., 2002; Goffredi et al., 1998; Goffredi et al., 1999).

Lamellibrachia luymesi van der Land and Nørrevang 1975 (Gardiner and Hourdez, 2003) is a vestimentiferan tubeworm associated with hydrocarbon seepage at 400–1000 m depths on the upper Louisiana slope of the Gulf of Mexico. It forms 1–2 m tall bush-like aggregations that can contain over one thousand individuals, and provide habitat for a wide variety of heterotrophic fauna (Bergquist et al., 2003; Cordes et al., 2005a; Cordes et al., 2005b). *L. luymesi* grows extremely slowly, and has a lifespan of at least 170–250 years (Bergquist et al., 2000; Fisher et al., 1997). Like all vestimentiferans, it requires sulfide for its survival and growth. Sulfide is produced within sediments at hydrocarbon seeps by microbial sulfate reduction coupled with oxidation of hydrocarbons such as methane and oil (Aharon and Fu, 2003; Boetius et al., 2000; Joye et al., 2004).

Whereas sulfide levels around the anterior gill-like plumes of adult tubeworms are generally less than 0.1 μmol l^{-1} (Freytag et al., 2001), levels in the sediment underlying them are typically greater than 1.5 mmol l^{-1} (Julian et al., 1999). *L. luymesi* obtains sulfide from the sediment using long, root-like, posterior extensions of its body (Freytag et al., 2001; Julian et

al., 1999). Large *L. luymesi* aggregations can have extensive networks of roots more than a meter deep in the underlying sediment (Fig. 1). These roots extend below the point at which the tubeworm tubes are attached to a carbonate rock substrate (Bergquist et al., 2002; Fisher et al., 1997).

Because sulfate and protons are the two major waste products of chemoautotrophic sulfide oxidation (Girguis et al., 2002; Goffredi et al., 1998; Goffredi et al., 1999), *L. luymesi* must possess mechanisms for eliminating sulfate and hydrogen ions. The hydrothermal vent tubeworm *Riftia pachyptila* has been relatively well studied, and has become a model for understanding vestimentiferan physiology. *R. pachyptila* does not grow a root, but uses its vascular plume to obtain sulfide and exchange other nutrients with its external environment (Arp et al., 1985). Its blood sulfate concentration is 22–25 mmol l^{-1} (Goffredi et al., 1999), which is lower than the seawater sulfate concentration of approximately 29 mmol l^{-1}. Thus *R. pachyptila* is thought to eliminate sulfate ions across its plume surface using membrane transporters that pump sulfate against the concentration gradient, consuming energy in the process (Goffredi et al., 1999). Likewise, *R. pachyptila* eliminates protons using a high concentration of proton-specific ATPases in its plume membrane, and this process represents a substantial metabolic cost for the tubeworm (Girguis et al., 2002; Goffredi and Childress, 2001).

Internal pH and sulfate concentrations of *L. luymesi* have not been previously reported. If they were similar to those of *R. pachyptila*, *L. luymesi* would also have to utilize active membrane transport to eliminate sulfate and hydrogen ions across its plume into the surrounding seawater. However, unlike *R. pachyptila*, *L. luymesi* could conceivably use its root for waste elimination. Microbial sulfate reduction depletes sulfate and hydrogen ions from the sediment pore-water surrounding *L. luymesi* roots, creating a favorable gradient for these ions to diffuse out of the roots. *L. luymesi* could avoid the high energetic demands of sulfate and proton elimination across its plume by passive transport of these ions across its roots.

L. luymesi could derive another important benefit from eliminating sulfate across its roots. In hydrocarbon rich sediments where *L. luymesi* are found, microbial sulfide production can become limited by sulfate availability (Arvidson et al., 2004; Joye et al., 2004). A number of authors have speculated that *L. luymesi* might sustain microbial sulfide production by 'irrigating' the sediments with sulfate (Cordes et al., 2005a; Cordes et al., 2003; Freytag et al., 2001; Julian et al., 1999). Cordes et al. (Cordes et al., 2005a) modeled the sulfide sources and demands of mature tubeworm aggregations and concluded that their demands could be satisfied over their entire lifespan only if individuals supplied sulfate across their roots into the sediment.

In this study, we measured the sulfate concentration and pH of *L. luymesi* body fluids and confirmed that elimination of these ions across the root surface would be energetically favorable in their natural habitat. We conducted laboratory experiments with live *L. luymesi* to measure sulfate and hydrogen ion elimination rates across their plume and root surfaces, and used anion transport inhibitors to begin to examine the molecular mechanism by which sulfate elimination occurs.

Materials and methods

Collection and maintenance of tubeworms

Small clumps of tubeworms *Lamellibrachia luymesi* about 50 cm long were collected from the site GC234, located at a depth of approximately 540 m on the upper Louisiana slope of the Gulf of Mexico (27°44.7′N, 91°13.3′W). The robotic claw of the Johnson Sea-Link submersible was used to grasp the carbonate rock to which the tubeworm clumps were attached to and place them in a temperature-insulated box for transport to the surface. After collection, the tubeworms were maintained on board the ship for up to 15 days in a cooler with cold (6°C) circulating seawater. Once a day, about one-third of the seawater in the cooler was replaced with clean seawater and the tubeworms were 'fed' for about 1 h by transferring them to a 20-l bucket containing a 500 μmol l^{-1} sodium sulfide solution in cold seawater. They were then returned to the maintenance cooler.

The tubeworms were transported in chilled seawater to the laboratory at the Pennsylvania State University where they were maintained at ambient pressure in specially designed aquaria inside cold rooms (6°C). The base of each aquarium was fitted with a polyvinylchloride (PVC) grating with holes drilled into it, which was connected to a hose that could be used to introduce sulfide under the sediment in the aquarium (Fig. 2). The grating was covered with a layer of crushed corals, above which was a ~10 cm layer of sediment collected from the seafloor of the Gulf of Mexico. The remainder of the aquarium was filled with synthetic seawater (SSW), made using Reef Crystals® (Aquarium Systems Inc., Mentor, OH, USA). The SSW in the aquariums was filtered and aerated using a flow-through aquarium filtration system, and about 10% of it was replaced with freshly made SSW once a week. The tubeworms were 'fed' twice a week by adding a stock solution containing 6 g of sodium sulfide dissolved in about 7 l of SSW through the PVC hose directly into the sediment below the tubeworms, at a rate of approximately 3 l h^{-1}.

Measurements of body fluid pH and sulfate concentrations

To obtain mixed body fluids (coelomic fluid and vascular blood), tubeworms were dissected within 2–3 h after they were collected from the seafloor. The seawater from their tubes was drained, following which they were cut at a point approximately 10 cm above the posterior ends of their tubes. The first few drops of blood were discarded because of the possibility of contamination with seawater, and the remaining blood was collected for analysis. The blood pH was measured using a MI-710 micro-combination pH electrode (Microelectrodes, Inc., Londondery, New Hampshire, USA) connected to a high-impedance Blood Gas Analyzer system (Cameron Instrument Company, Port Aransas, Texas, USA).

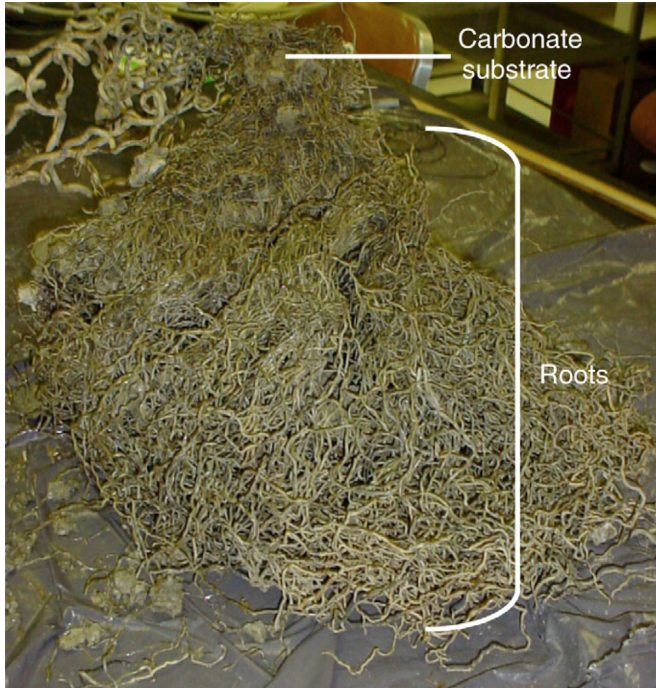

Fig. 1. The extensive tubeworm roots of an aggregation that was excavated and collected using the Bushmaster sampling device (Bergquist et al., 2002; Cordes et al., 2005b). This photograph shows roots that are about 1 m long, extending below the carbonate rock substrate. The roots were observed to break during collection and we believe they might have been even longer in the intact aggregation (C.R.F., personal observation). Photo courtesy of Dr E. E. Cordes.

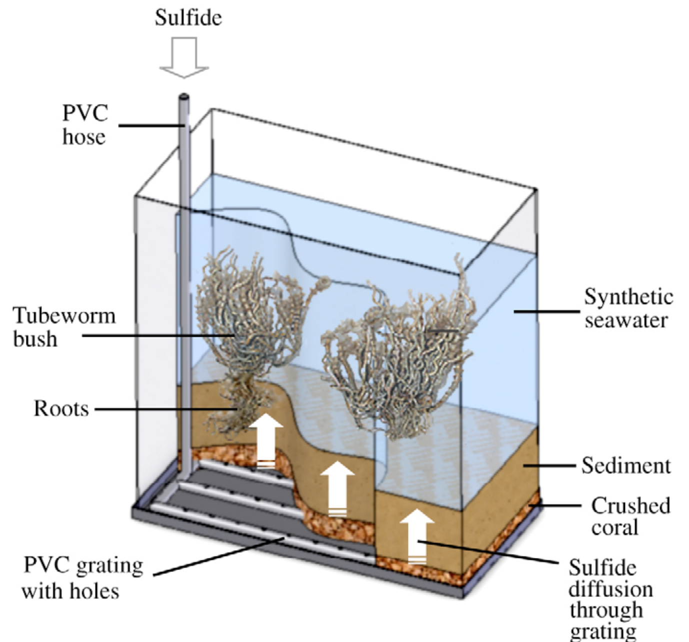

Fig. 2. Schematic representation of a tubeworm maintenance aquarium. Sulfide dissolved in synthetic seawater (SSW) was introduced into the polyvinylchloride (PVC) hose, which was connected to PVC grating. The sulfide diffused out from the grating through the crushed coral and sediment layers. The aquarium was filled with SSW, which was kept aerated and filtered.

The electrode was calibrated using IUPAC standards of pH 6.86±0.01 and 7.41±0.01 (Radiometer Analytical, Villeurbanne, CX, France). Hemoglobins and other proteins were then removed from the blood by adding a 1:1 volume of methanol, following which the solution was centrifuged at 3000 ***g*** for 10 min (Goffredi et al., 1999). The pellet was discarded and the supernatant was diluted to 25 ml using double-distilled deionized water. The sulfate concentration in the supernatant was determined using turbidimetric analysis, details of which are described below.

Live tubeworm experiments

Sulfide spontaneously oxidizes to form sulfate in the presence of oxygen, so sulfate excretion by tubeworms could not be measured while simultaneously exposing the animals to sulfide. Instead, tubeworms were first exposed to sulfide so that they accumulated bound sulfide in their blood, and then transferred to experimental chambers without sulfide to measure sulfate and proton excretion rates. The tubeworms used for our experiments were typically 30–50 cm long, with wet tissue masses of between 2 and 5 g. Assuming that these tubeworms contained 14 ml of blood, and the bound sulfide concentrations in their blood were 150–170 μmol l^{-1} (Freytag et al., 2001), sulfide oxidation would produce approximately 0.15–0.8 μmoles of sulfate in total. This amount of sulfate would be undetectable in seawater, which has a sulfate concentration of 29 mmol l^{-1}. Thus a sulfate-free artificial seawater (SF-ASW) medium was used when measuring tubeworm sulfate elimination rates. The SF-ASW contained 500 mmol l^{-1} sodium chloride, 9 mmol l^{-1} potassium chloride, 9.3 mmol l^{-1} calcium chloride, 48.5 mmol l^{-1} magnesium chloride, and 2.5 mmol l^{-1} sodium bicarbonate. The SF-ASW had a salinity of 36‰ and its pH was adjusted to 8.0 using a 0.5 mmol l^{-1} sodium hydroxide solution.

Sulfate elimination rates of freshly collected tubeworms were determined as a reference for comparison with rates of tubeworms maintained in aquariums. These rates were measured in a cold room (6–8°C) on board the ship using tubeworms that had been collected from the seafloor less than 24 h previously. Seawater was drained from their tubes, and they were soaked in SF-ASW for 20 min to minimize the sulfate carried over by their tubes into the experimental chambers. One or two animals were then inserted into a polycarbonate respirometer, which was filled with SF-ASW and re-circulated continuously using a peristaltic pump. At the end of 48 h, the solution from the respirometer was collected and its sulfate concentration was determined. The tissue wet masses of these tubeworms were estimated from the volume of seawater their soft tissues displaced in a 100 ml graduated cylinder, as there was no shipboard balance available.

The remaining experiments were performed with animals maintained in aquaria for a period of 1–15 months. Before a

tubeworm was used for an experiment, it was transferred from the maintenance aquarium to a 2-l glass graduated cylinder containing SSW, where its plume coloration and reflexes were observed during a period of 15–20 h to ensure that it appeared to be in good physiological condition. A tubeworm was selected for an experiment if it routinely extended its plume outside its tube, its plume was bright red in color, and if it reacted to a sharp knock on the glass cylinder by rapidly withdrawing its plume back into its tube. Before an experiment, tubeworms were 'fed' by incubating them with a 500 μmol l^{-1} solution of sodium sulfide in SSW for 48 h. SSW was drained from their tubes, and they were soaked in SF-ASW for 20 min. They were then inserted into a 'split-chamber' polycarbonate respirometer, which enabled separation of their anterior and posterior halves into distinct, watertight chambers (Freytag et al., 2001; Girguis et al., 2002). These chambers contained SF-ASW that was continuously re-circulated using a peristaltic pump. A headspace of air supplemented the oxygen dissolved in the SF-ASW in the anterior chamber. Since the concentration of oxygen in air is about 25 times higher than that dissolved in seawater at 6°C (Carpenter, 1966), circulation of the SF-ASW through the headspace of air was intended to prevent hypoxic conditions. The volume of the headspace was approximately 50 ml for small worms (up to 3.5 g wet mass) and approximately 100 ml for larger worms. At the end of 48 h, the solutions from the plume and root chambers were collected and their sulfate concentrations and pH were determined. The worm was then removed from its tube, and its wet mass was measured.

'Fed' tubeworms were exposed to a sulfate-free medium during the above experiments. As a control for sulfate loss from the animal driven by the large sulfate gradient between their tissues and the SF-ASW, we conducted experiments with 'starved' tubeworms. These experiments were performed using the same procedure described above for the 'fed' tubeworms, except the tubeworms were incubated in SSW without sulfide for at least 96 h prior to measuring their sulfate and proton elimination rates.

The effects of inhibitors on tubeworm sulfate and proton elimination rates were measured in a three-part experiment. The inhibitors we used were the general anion exchange inhibitors DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) and SITS (4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid), and the potent chloride transport inhibitor NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid) (Cabantchik and Greger, 1992). In these experiments, the procedures used for 'feeding' tubeworms and measuring elimination rates were the same as those described above with 'fed' tubeworms. First, a tubeworm was 'fed' sulfide for 48 h and its 'baseline' sulfate and proton elimination rates were measured. It was then removed from the experimental chamber and 'fed' for another 48 h, following which it was incubated with an inhibitor solution for 3 h. Its 'inhibitor-exposed' sulfate and proton elimination rates were then determined. Finally, it was 'fed' for another 48 h, after which its 'post-exposure' sulfate and proton elimination rates were measured. The wet mass of the tubeworm was then determined as above. The various inhibitor solutions used for these experiments were 0.2 mmol l^{-1} DIDS, 0.4 mmol l^{-1} SITS and 0.1 mmol l^{-1} NPPB. To make an inhibitor solution, either DIDS, SITS or NPPB was first dissolved in 250 μl of dimethyl sulfoxide (DMSO) and then mixed with 250 ml of SSW so that the final concentration of DMSO in the solution was 0.1% by volume. Control experiments were performed in which tubeworms were exposed to a 0.1% solution of DMSO in SSW instead of an inhibitor solution. DIDS, SITS, NPPB and DMSO were all purchased from Sigma Chemical Company, St Louis, MO, USA.

Measurement of sulfate elimination rates

Sulfate concentrations of solutions collected from plume and root chambers in the experiments described above were measured using barium chloride turbidimetry, and all samples were analyzed in duplicate. Specifically, one SulfaVer® reagent packet (Hach Chemical Company, Loveland, CO, USA) was added to 25 ml of the sample, and the solution was vortexed. The reaction was allowed to proceed for exactly 5 min, after which the absorbance of the sample was measured at 450 nm using a Beckman DU-64 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). The sulfate concentration was calculated from a standard curve generated using serial dilutions of a 2500 mg l^{-1} sulfate standard (Hach Chemical Company) diluted using SF-ASW. As the sulfate concentration in the SF-ASW introduced into the plume and root chambers at the beginning of each experiment was empirically determined to be zero, the entire sulfate measured in the final samples was assumed to have been released by the tubeworms. Sulfate elimination rates were calculated from sulfate measurements, incubation times in chambers with SF-ASW and tubeworm mass.

Measurement of proton elimination rates

pH of solutions collected from experimental chambers were measured to estimate the amount of protons released by tubeworms. The SF-ASW was first equilibrated with air by stirring it continuously for 48 h at 6°C with a headspace of air above it, to ensure that it reached a steady pH value. A 50 ml sub-sample of the SF-ASW was kept aside in a sealed bottle for subsequent analysis of initial pH and buffering capacity. The SF-ASW was circulated with tubeworms inside split-respirometers for a period of 48 h, at the end of which pH of the final plume and root chamber samples were measured. The pH of the initial SF-ASW was measured at the same time, and its buffering capacity was determined empirically by titration with a 0.1 mol l^{-1} hydrochloric acid solution. The titration curve was used to determine the proton release into the plume or root chambers necessary to cause the observed differences in pH between the initial and final SF-ASW solutions. Control experiments in which SF-ASW equilibrated as above was circulated in split-respirometers without tubeworms showed that there was no change in pH of SF-ASW in the absence of tubeworms. Proton elimination rates of tubeworms were calculated from proton measurements, incubation times in chambers with SF-ASW, and tubeworm mass.

Data analysis and statistics

The program MINITAB (Minitab Inc., State College, PA, USA) was used for all statistical analyses. Student's *t*-tests were used to compare sulfate and proton excretion rates between 'fed' and 'starved' animals, and also for inhibitor studies. When the *a priori* expectation was that a treatment would reduce the rate of sulfate or proton release, one-sided *t*-tests were used. For inhibitor experiments where the same individual was used for more than one treatment, paired *t*-tests for were used for comparisons. Correlations between parameters were analyzed using the Pearson method.

Results

Body fluid parameters

Mixed blood from freshly collected *L. luymesi* had a sulfate concentration of 23.10±1.76 mol l^{-1} (mean ± s.d.; N=15), and a pH of 7.12±0.14 (mean ± s.d.; N=10).

Laboratory maintenance of tubeworms

Tubeworms were successfully maintained alive in aquaria in the laboratory at 6°C and under atmospheric pressure. Most of the tubeworms extended their plumes outside their tubes when sulfide-rich SSW was being introduced into their aquarium. The trophosomes of tubeworms maintained in aquaria were observed to be light to dark green in color, indicating the presence of elemental sulfur reserves (Pflugfelder et al., 2005). Some tubeworms incurred damage to their roots during collection from the seafloor, and a majority of these worms added new root tube material to the posterior ends at which their root tubes had been broken. Laboratory-maintained tubeworms eliminated sulfate at a rate of 0.457±0.176 μmoles g^{-1} wet mass h^{-1} (mean ± s.d., N=32), which was not significantly lower (P=0.304, one-sided *t*-test) than the rate at which freshly collected tubeworms eliminated sulfate (mean ± s.d. = 0.509±0.144 μmoles g^{-1} wet mass h^{-1}, N=3).

'Fed' tubeworms: sulfate and proton elimination rates

L. luymesi eliminated both sulfate and hydrogen ions at significantly higher rates across their roots than across their plumes (Table 1; sulfate: P=0.0001, N=32; protons: P=0.0003, N=12; two-sided paired *t*-tests). Plume sulfate elimination rates were weakly correlated with root sulfate elimination rates (Fig. 3A; R=0.31, P=0.066). Conversely, plume proton elimination rates were strongly correlated with root proton elimination rates (Fig. 3B; R=0.91, P<0.0001). Proton elimination rates were negatively correlated with sulfate elimination rates, when measured across the plumes (Fig. 4A; R=–0.69, P=0.012) and across the roots (Fig. 4B; R=–0.67, P=0.017). Total proton elimination rates (plume and root rates combined) were also negatively correlated with total sulfate elimination rates (Fig. 4C; R=–0.78, P=0.003).

Comparison between 'starved' and 'fed' tubeworms

'Starved' tubeworms eliminated sulfate across their plumes at a slightly lower average rate than 'fed' tubeworms, but this difference was not statistically significant (Table 1; P=0.143, one-sided *t*-test, d.f.=26). However, 'starved' tubeworms eliminated sulfate across their roots at a substantially lower rate than 'fed' tubeworms, and this difference was highly significant (Table 1; P<0.0001, one-sided *t*-test, d.f.=26). 'Starved' tubeworms had similar levels of proton elimination as 'fed' tubeworms, across their plumes (Table 1; P=0.771, one-sided *t*-test, d.f.=9) as well as across their roots (P=0.530, one-sided *t*-test, d.f.=9).

The effect of inhibitors on sulfate and proton elimination rates

Control experiments in which tubeworms were exposed to 0.1% DMSO showed that this treatment did not significantly decrease sulfate elimination rates either across the plumes or roots of tubeworms (Fig. 5A; plume: P=0.447; root: P=0.945; one-sided paired *t*-tests; N=5). However, exposure to DIDS or SITS caused a significant decrease in the sulfate elimination rates across roots of *L. luymesi*, as compared to 'baseline' values (Fig. 5B,C; DIDS: P=0.045, one-sided paired *t*-test, N=7; SITS: P=0.040, one-sided paired *t*-test, N=6). Neither inhibitor significantly decreased sulfate elimination rates across plumes of these animals (DIDS: P= 0.229, one-sided paired *t*-test, N=7; SITS: P=0.188, one-sided paired *t*-test, N=6). DIDS appeared to have an irreversible effect on sulfate transport across roots, as sulfate elimination rates of 'post-exposure' animals were significantly lower than that of 'baseline' values (P=0.015; one-sided paired *t*-test, N=7). However, 'post-exposure' sulfate elimination of SITS-exposed tubeworms was not significantly lower than the 'baseline values' (P=0.779,

Table 1. *Sulfate and proton elimination by tubeworms over a period of 48 h*

	Sulfate elimination rate (μmol h^{-1} g^{-1} wet mass)		Proton elimination rate (μequivalents h^{-1} g^{-1} wet mass)	
Experiment type	Plume	Root	Plume	Root
Fed	0.163±0.014^{a} (32)	0.276±0.023^{a} (32)	0.138±0.040^{a} (12)	0.416±0.080^{c} (12)
Starved	0.129±0.027^{a} (12)	0.094±0.029^{b} (12)	0.174±0.027^{a} (5)	0.427±0.029^{c} (5)

Tubeworms were either 'fed' sulfide for a period of 48 h prior to the experiment, or were 'starved' of sulfide for a period of at least 96 h prior to the experiment.

Values are means ± s.e.m. (N values are given in parentheses). Different superscript letters after a value indicate a significant difference, using the Bonferroni corrected significance level of 0.0025.

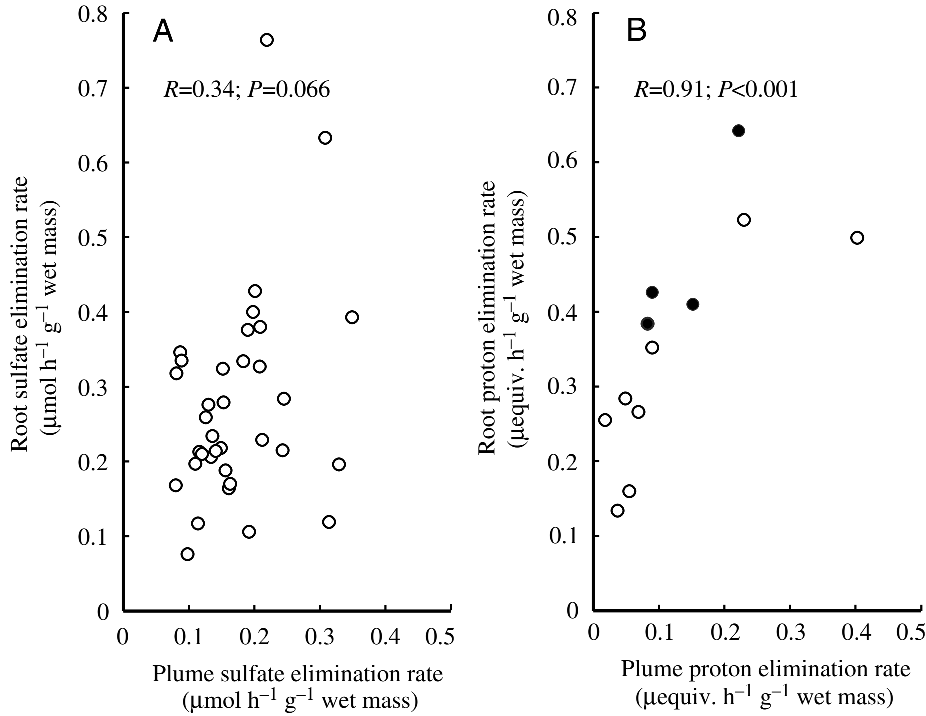

Fig. 3. Relation between (A) sulfate (μmol h^{-1} g^{-1} wet mass), and (B) protons (μequiv. h^{-1} g^{-1} wet mass) eliminated across plumes and roots of individual tubeworms exposed to sulfide for 48 h prior to the measurement. Closed circles in B represent animals that might have experienced hypoxic conditions and eliminated protons from anaerobic metabolism.

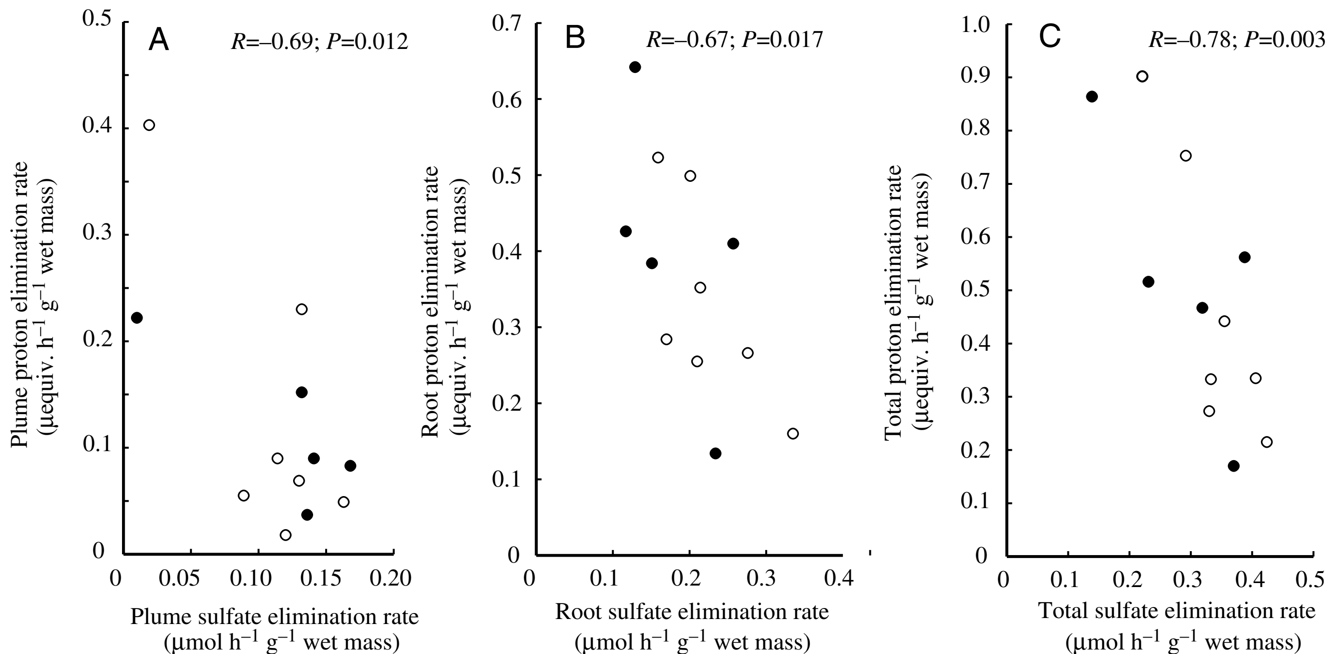

Fig. 4. Relation between (A) plume, (B) root and (C) total (plume and root combined) proton and sulfate eliminated from individual tubeworms exposed to sulfide for 48 h prior to the experiment. Closed circles represent animals that might have experienced hypoxic conditions and eliminated protons from anaerobic metabolism.

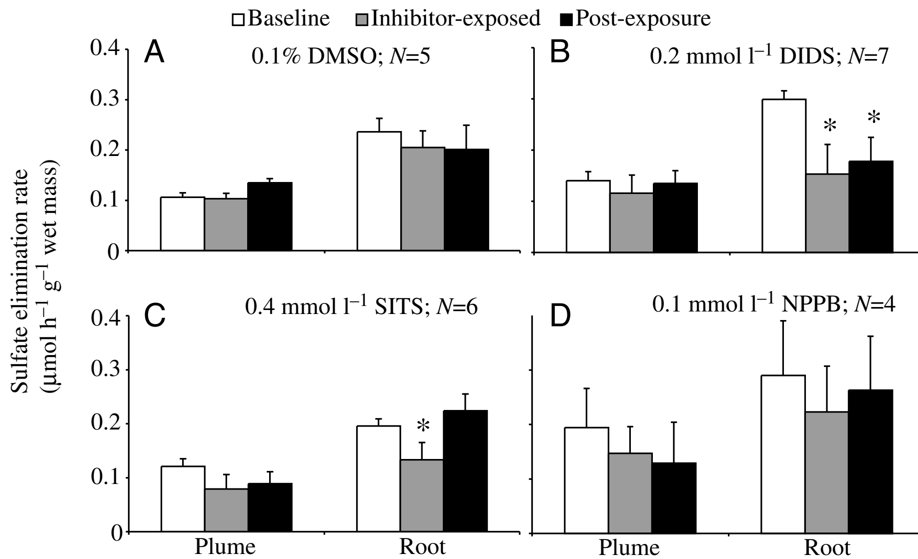

Fig. 5. A summary of the effect of the membrane transport inhibitors and the solvent (DMSO) on sulfate elimination across plumes and roots of *L. luymesi*. An asterisk indicates that the treatment caused significant (P<0.05) inhibition of sulfate elimination.

one-sided paired t-test, N=6). Exposure to NPPB did not cause a significant decrease in sulfate elimination across either roots or plumes of tubeworms (Fig. 5D; plume: P=0.317; root: P=0.327, one-sided paired t-tests; N=4). None of the above inhibitors had a significant effect on proton release across either plumes or roots of tubeworms (P>0.1 for all inhibitors).

Discussion

Vestimentiferan tubeworms possess a variety of specific physiological adaptations to sustain their obligate symbiosis with sulfide-oxidizing autotrophic bacteria. They successfully support carbon fixation by their symbionts by obtaining sulfide, oxygen and inorganic carbon from their environments and supplying these nutrients to their symbionts *via* their circulating vascular system (Arp et al., 1985; Cavanaugh et al., 1981; Childress et al., 1984; Freytag et al., 2001). In addition, they ensure that bacterial sulfide oxidation is not inhibited by a build-up of end products, namely, sulfate and hydrogen ions. The hydrothermal vent tubeworm *R. pachyptila* eliminates these waste ions across its plume surface (Girguis et al., 2002; Goffredi et al., 1999). By contrast, we found that the hydrocarbon seep tubeworm *L. luymesi* primarily used its roots to excrete these waste ions (Table 1). The main goal of this study was to identify the mechanism and location of sulfate and proton elimination. Owing to experimental constraints, we did not simultaneously expose tubeworms to sulfide while measuring their sulfate and proton elimination rates. Thus the rates we report are probably lower than the rates characteristic of *L. luymesi* in their natural habitat.

The plume is considered to be the primary exchange organ for hydrothermal vent vestimentiferans because of its large surface area, extensive vascularization, and short diffusion distances between blood vessels and the external environment (Arp et al., 1985; Gardiner and Jones, 1993). Sulfate and hydrogen ions are produced by symbionts located in the trophosome (Cavanaugh et al., 1981) and in the case of *R. pachyptila* are carried by the vascular blood to the plume, where they are probably eliminated across the plume epithelium (Goffredi et al., 1999). Unlike *R. pachyptila*, vascular connections between the trophosome and the body wall have been reported for *L. luymesi* (Gardiner and Jones, 1993; van der Land and Nørrevang, 1977), which might provide a direct route for transfer of ions between the two tissues in the seep species. Additionally, waste ions might be transferred to the coelomic fluid, which is in equilibrium with the vascular blood for most ions, and is contained within two cavities between the trophosome and the body wall (Childress et al., 1984; Jones, 1981). The coelomic fluid might mediate transfer of ions from the trophosome to the body wall, where they might be eliminated across the body wall epithelium. In the case of *L. luymesi*, the root body wall might comprise a significant exchange surface. For example, a mature *L. luymesi* tubeworm that is about 1 m tall above its point of attachment to the carbonate substrate can have a root that is about the same length (1 m) (Cordes et al., 2005a). The root can be approximated as a cylinder with 1.5 mm diameter, having a surface area of 47 cm^2. The mass of a 1 m tall *L. luymesi* individual is about 6 g, based on the mass to length conversion described by Cordes et al. (Cordes et al., 2003). The plume surface area of *L. luymesi* has not been measured, but using measurements made on a vestimentiferan of similar morphology, the long-skinny morphotype of *Ridgeia piscesae*, the plume surface area of a 6 g worm is approximately 53 cm^2 (A. C. Anderson, J. F. Flores, S. Hourdez, manuscript submitted). Thus the surface areas of the root and plume are of the same order of magnitude in *L. luymesi* and both are probably important gas and ion exchange surfaces.

From an energetic standpoint, the root rather than the plume of *L. luymesi* might be the favorable exchange surface for sulfate and hydrogen ion elimination. Passive facilitated diffusion can mediate membrane transport of an ion in the

direction of its electrochemical gradient, whereas energetically expensive active transport is required to transport an ion against its gradient (Byrne and Schultz, 1994). In this study, we found that *L. luymesi* body fluids have an average sulfate concentration of 23 mmol l^{-1} and an average pH of 7.12. We did not measure intracellular pH and sulfate concentrations of plume or root epithelial cells. However, *R. pachyptila* intracellular pH was very similar to that of its body fluids (Goffredi et al., 1999), and we assumed the same for *L. luymesi*. *L. luymesi* cells probably have a resting membrane potential of –70 mV, which is typical for most animal cells (Lodish et al., 2000). Based on the Nernst equation (Hille, 1992), the electrochemical gradient would favor proton efflux from *L. luymesi* only when external pH values were greater than 8.4. Similarly, sulfate efflux would be favorable if the extracellular sulfate concentration were lower than the intracellular concentration. In their natural habitat, *L. luymesi* plumes are bathed in seawater that has a sulfate concentration of about 29 mmol l^{-1} and a pH of about 7.7, whereas their roots are surrounded by sediment pore-water in which sulfate and hydrogen ions are depleted as a result of microbial sulfate reduction (Aharon and Fu, 2000; Arvidson et al., 2004). For example, at sediment depths greater than 20 cm at hydrocarbon-rich sites, pore-water sulfate concentrations can vary between 0 and 18 mmol l^{-1} and pH can vary between 7.9 and 9.0 (Aharon and Fu, 2000). Therefore, *L. luymesi* could possibly eliminate sulfate and hydrogen ions using passive facilitated diffusion across its root epithelial membrane, whereas it would require energetically expensive ion pumps to eliminate these ions across its plume membrane.

Sulfate elimination from experimental animals

In order to measure sulfate elimination rates across plume and root surfaces of *L. luymesi*, we incubated them for 48 h with a sulfate-free medium inside split-chamber respirometers (Freytag et al., 2001; Girguis et al., 2002). We performed our experiments with either ‘fed’ tubeworms that had previously been exposed to sulfide for 48 h, or ‘starved’ tubeworms that had previously been deprived of sulfide for at least 96 h. During the experiments, ‘fed’ tubeworms probably eliminated sulfate derived from oxidation of sulfide carried in their blood. Conversely, ‘starved’ tubeworms had minimal bound sulfide in their blood (Freytag et al., 2001) and were unlikely to eliminate sulfate derived from sulfide oxidation. Moreover, since we exposed these tubeworms to a sulfate-free external medium, a portion of the sulfate they eliminated during the experimental period was probably driven by diffusion not mediated by specific membrane transporters. Goffredi et al. (Goffredi et al., 1999) found that the sulfate level in the coelomic fluid of *R. pachyptila* deprived of sulfide for 48–72 h was just 5 mmol l^{-1} lower than that of sulfide-exposed tubeworms. Based on this, we could assume that ‘fed’ and ‘starved’ tubeworms faced similar gradients when exposed to SF-ASW, and eliminated similar levels of sulfate by unmediated diffusion. This allowed us to estimate the rate of sulfate elimination derived from sulfide oxidation alone, by subtracting sulfate elimination rates of ‘starved’ tubeworms from those of ‘fed’ tubeworms. In doing so, we found that 85% of the total sulfate derived from sulfide oxidation was eliminated across the root surface. There was a substantial and statistically significant difference (0.182 μmol h^{-1} g^{-1} wet mass) between the average sulfate elimination rates across roots of ‘fed’ and ‘starved’ tubeworms (Table 1). Conversely, there was a small and statistically insignificant difference (0.033 μmol h^{-1} g^{-1} wet mass) between the average sulfate elimination rates across plumes of ‘fed’ and ‘starved’ animals.

In their natural habitat, tubeworms might occasionally experience sulfate-free conditions across their roots (Arvidson et al., 2004). However, their plumes are always exposed to seawater containing 29 mmol l^{-1}. It appears that under our experimental conditions *L. luymesi* eliminated sulfate across their plumes primarily by unmediated diffusion, while they eliminated most of the sulfate derived from sulfide oxidation across their roots. The extent of plume sulfate elimination would depend on the gill surface area and the sulfate concentration gradient, whereas the extent of root sulfate elimination would depend on the amount of sulfide oxidation the tubeworm underwent during the experimental time period. Plume surface area may differ between individual tubeworms and is not likely to correlate with the rate of sulfide oxidation within the animal. This may explain the relatively weak correlation we found between plume and root sulfate elimination rates of individual tubeworms (Fig. 3A).

The mechanism of sulfate release

We treated live *L. luymesi* with inhibitors of membrane anion transport in order to deduce the mechanism of sulfate elimination. The anion exchange inhibitors, DIDS and SITS significantly inhibited root sulfate elimination (Fig. 5B,C), but had no significant effect on plume sulfate elimination. Both DIDS and SITS can bind reversibly, but are also known to have covalent binding capacities leading to irreversible effects (Cabantchik and Greger, 1992). We found that with respect to *L. luymesi* sulfate transport, DIDS was the more potent of the two inhibitors and had an irreversible effect, whereas SITS was less potent and appeared to bind reversibly (Fig. 5B,C). Probes of anion transport, such as DIDS and SITS often have broad specificities (Cabantchik and Greger, 1992), but sensitivity to these inhibitors indicates the presence of an anion antiport system (Gerencser et al., 1996). Thus *L. luymesi* roots most probably contain sulfate exchangers through which they mediate the excretion of this ion, whereas their plumes do not appear to have this mechanism. Similar to our findings, Goffredi et al. (Goffredi et al., 1999) did not find evidence of DIDS or SITS-sensitive sulfate exchangers in *R. pachyptila* plumes.

DIDS and SITS-sensitive sulfate transporters are found in a variety of taxonomic groups including invertebrates (Gerencser et al., 1996; Gerencser et al., 1999; Shimuzu and Bradley, 1994), fish (Renfro, 1999; Renfro and Pritchard, 1983) and mammals (Markovich, 2001; Pritchard and Renfro, 1983). DIDS and SITS inhibit the well-studied mammalian band-3

anion exchanger from red blood cell membranes that can mediate transport of chloride, bicarbonate and sulfate (Markovich, 2001). In marine organisms, sulfate is commonly exchanged with chloride and bicarbonate that are abundant in seawater (Gerencser et al., 1996; Gerencser et al., 1999; Renfro, 1999). Thus, it is plausible that *L. luymesi* roots have an anion transporter that exchanges sulfate ions for either chloride or bicarbonate ions. To examine whether *L. luymesi* roots eliminate sulfate using sulfate–chloride antiports, we analyzed sensitivity of root sulfate transport to NPPB, a potent chloride transport inhibitor (Cabantchik and Greger, 1992; Culliford et al., 2002; Gelband et al., 1996). Interestingly, NPPB significantly affects chloride transport across the bacteriocyte membrane in the closely related tubeworm *R. pachyptila* (de Cian et al., 2003). We found that NPPB did not have a significant effect on sulfate transport across *L. luymesi* roots (Fig. 5D), indicating that sulfate transport across the *L. luymesi* root membrane might not occur *via* a sulfate–chloride exchanger.

Alternately, sulfate elimination across *L. luymesi* roots might occur *via* sulfate–bicarbonate exchangers. Sulfate-bicarbonate antiports are found in several organisms including rats (Pritchard and Renfro, 1983), teleosts (Renfro, 1999) and lobsters (Gerencser et al., 1999), and are often sensitive to DIDS and SITS. For *L. luymesi*, uptake of bicarbonate in lieu of sulfate elimination across its roots is reasonable in light of several facts. Bicarbonate levels in sediment pore-water surrounding tubeworms roots are high (Joye et al., 2004; MacDonald, 1998). Bicarbonate is produced in the sediments as a byproduct of sulfate reduction coupled with hydrocarbon oxidation, the same process that produces sulfide (Sassen et al., 1994; Valentine, 2002). Therefore, bicarbonate uptake by tubeworms from the sediment could enhance sulfide production due to end-product removal. Further, tubeworms could utilize the bicarbonate they take up across their roots for carbon fixation by their symbionts. *R. pachyptila* takes up inorganic carbon in the form of carbon dioxide by diffusion across its plume surface, facilitated by the high partial pressures of CO_2 in acidic vent waters (Childress et al., 1993; Goffredi et al., 1997). However, *L. luymesi* plumes are bathed in seawater with pH of about 7.7 (Aharon and Fu, 2000), at which pCO_2 levels are negligible. *L. luymesi* might obtain at least part of its inorganic carbon from the sediment pore-water across its roots. This is consistent with the fact that *L. luymesi* tissues often have depleted stable carbon isotope values that reflect incorporation of inorganic carbon derived from oxidized methane and crude oil (Kennicutt, II et al., 1992; Roberts and Aharon, 1994). Finally, carbonate encrustation of tubeworm root tubes could reduce their permeability to sulfate and sulfide. None of the several thousand *L. luymesi* that have been collected to date had carbonate deposited directly on the root tube surface, although root-balls of the aggregations are often partially embedded in carbonate (Fig. 1) (Cordes et al., 2005a). It is plausible that *L. luymesi* limit carbonate precipitation directly onto their root tubes by taking up bicarbonate and releasing protons across their roots, thereby decreasing pore-water bicarbonate concentrations and pH (Cordes et al., 2005a).

Proton elimination from experimental animals

We found that 'fed' tubeworms eliminated protons across their roots on average three times faster than across their plumes (Table 1). The root proton elimination rates of individual tubeworms were strongly correlated with plume elimination rates (Fig. 4B), and the relationship had a slope of approximately 2.5. This indicates that on an average, for every proton eliminated across the plume, 2.5–3 protons were eliminated across the root. Overall, approximately 67% of the total proton elimination occurred across the roots.

In our study, we found that 'fed' and 'starved' tubeworms eliminated protons at very similar rates (Table 1). Owing to experimental constraints, we were unable to expose the tubeworms to sulfide during the measurement of proton flux. Thus our proton flux values were rather low, and in the same order of magnitude as 'pre-sulfide' exposure rates of *L. luymesi* measured by Girguis et al. (Girguis et al., 2002). Girguis and co-workers found that proton elimination by *R. pachyptila* ceased just 1–2 h after exposure to sulfide was terminated. Therefore, it is likely that in our experiments neither 'fed' nor 'starved' tubeworms eliminated a significant amount of protons derived from sulfide oxidation. Instead, the proton flux we measured may have been derived from heterotrophic processes.

Moreover, we included a headspace of air in the anterior chamber to prevent hypoxic conditions during our experiments. However, *post-hoc* calculations based on heterotrophic oxygen consumption rates of *L. luymesi* (Freytag et al., 2001) indicated that 3–3.5 g worms might have consumed all the oxygen available to them in the first 30–35 h of the 48 h experiment, and therefore might have produced protons as a result of anaerobic metabolism. In Figs 3 and 4, we have indicated animals that might have experienced hypoxia using different symbols. We observed no apparent differences with respect to the proton elimination patterns of these animals compared to those that did not experience hypoxic conditions, and the trends we report here do not change if we omit these animals from the analyses.

The relation between proton and sulfate elimination

Sulfate transport is dependent on proton gradients in a number of different organisms (Gerencser et al., 1996; Yildiz et al., 1994). Sulfate transport could also be directly coupled with proton transport through proton-sulfate symports (Leustek and Saito, 1999; Renfro and Pritchard, 1983). If this type of transporter were used by *L. luymesi* for sulfate elimination, we would expect to see a positive correlation between sulfate and proton elimination rates of individual tubeworms. By contrast, we observed a significant negative correlation between these rates (Fig. 4). Moreover, inhibition of sulfate elimination by DIDS and SITS did not affect proton elimination across *L. luymesi* roots. This combined evidence suggests that proton and sulfate elimination might not be coupled in *L. luymesi*. The negative correlation between proton and sulfate elimination

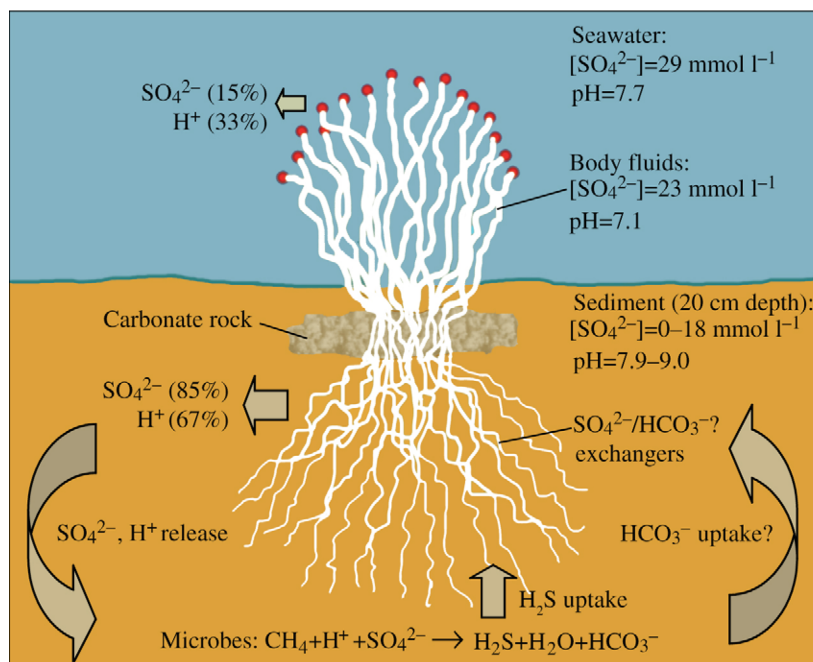

Fig. 6. A schematic representation of an aggregation of *L. luymesi*, depicting a nutrient exchange model for this species. Seawater and sediment pore-water sulfate concentration and pH are from Aharon and Fu (Aharon and Fu, 2000). The equation for microbial sulfate reduction shows sulfate reduction coupled with methane oxidation. However, sulfate reduction coupled with higher molecular mass hydrocarbons can also occur (Joye et al., 2004). Bicarbonate uptake across roots is suggested, but has not been directly demonstrated in this study.

rates is difficult to explain in terms of known metabolic or membrane processes. Further studies that examine membrane transport in *L. luymesi* in more detail are needed before a viable explanation can be provided.

Conclusion

Based on our previous knowledge about *L. luymesi* and the results of this study, we propose a conceptual model for nutrient uptake and waste elimination processes in *L. luymesi* (Fig. 6). In this study, we determined that body fluids of *L. luymesi* tubeworms have average sulfate concentrations of 23 mmol l^{-1} and an average pH of 7.12. This indicates that electrochemical gradients favor elimination of both sulfate and protons by passive facilitated diffusion across the root surface into the surrounding sediment. Therefore from an energetic perspective, it would be economical for *L. luymesi* to eliminate sulfate and hydrogen ions across its roots. Consistent with this, we found that under laboratory conditions *L. luymesi* eliminate approximately 85% of the sulfate produced by sulfide oxidation and approximately 67% of the protons produced by a combination of metabolic processes, across their roots. Our results also suggest that sulfate transport across the root membrane might occur *via* an antiport that exchanges sulfate for bicarbonate ions. Elimination of sulfate and protons across the roots into the surrounding sediment pore-water might ensure that sulfide production in the sediment around the roots would not be sulfate limited (Cordes et al., 2005a). Moreover, if bicarbonate uptake across the roots does occur, it might supplement *L. luymesi* inorganic carbon uptake and help to prevent carbonate precipitation on its root tubes.

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References

Aharon, P. and Fu, B. (2000). Microbial sulfate reduction rates and sulfur and oxygen isotope fractionations at oil and gas seeps in deepwater Gulf of Mexico. *Geochim. Cosmochim. Acta* **64**, 233-246.

Aharon, P. and Fu, B. (2003). Sulfur and oxygen isotopes of coeval sulfate-sulfide in pore fluids of cold seep sediments with sharp redox gradients. *Chem. Geol.* **195**, 201-218.

Arp, A. J., Childress, J. J. and Fisher, C. R. (1985). Blood gas transport in *Riftia pachyptila*. *Biol. Soc. Wash. Bull.* **6**, 289-300.

Arvidson, R. S., Morse, J. W. and Joye, S. B. (2004). The sulfur biogeochemistry of chemosynthetic cold seep communities, Gulf of Mexico, USA. *Mar. Chem.* **87**, 97-119.

Bergquist, D. C., Williams, F. M. and Fisher, C. R. (2000). Longevity record for deep-sea invertebrate: the growth rate of a marine tubeworm is tailored to different environments. *Nature* **403**, 499-500.

Bergquist, D. C., Urcuyo, I. A. and Fisher, C. R. (2002). Establishment and persistence of seep vestimentiferan aggregations on the upper Louisiana slope of the Gulf of Mexico. *Mar. Ecol. Prog. Ser.* **241**, 89-98.

Bergquist, D. C., Ward, T., Cordes, E. E., McNelis, T., Howlett, S., Kosoff, R., Hourdez, S., Carney, R. and Fisher, C. R. (2003). Community structure of vestimentiferan-generated habitat islands from Gulf of Mexico cold seeps. *J. Exp. Mar. Biol. Ecol.* **289**, 197-222.

Boetius, A., Ravenschlag, K., Schubert, C. J., Rickert, D., Widdel, F., Gieseke, A., Amann, R., Jorgensen, B. B., Witte, U. and Pfannkuche, O. (2000). A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**, 623-625.

Brusca, R. C. and Brusca, G. J. (2002). *Invertebrates*. Sunderland, MA: Sinauer Associates,.

Byrne, J. H. and Schultz, S. G. (1994). *An Introduction to Membrane Transport and Bioelectricity*. New York: Raven Press.

Cabantchik, Z. I. and Greger, R. (1992). Chemical probes for anion transporters of mammalian cell membranes. *Am. J. Physiol.* **265**, C803-C827.

Carpenter, J. H. (1966). New measurements of oxygen solubility in pure and natural water. *Limnol. Oceanogr.* **11**, 264-277.

Cavanaugh, C. M., Gardiner, S. L., Jones, M. L., Jannasch, H. W. and Waterbury, J. B. (1981). Prokaryotic cells in the hydrothermal vent tubeworm *Riftia pachyptila* Jones: possible chemoautotrophic symbionts. *Science* **213**, 340-342.

Childress, J. J., Arp, A. J. and Fisher, C. R. (1984). Metabolic and blood characteristics of the hydrothermal vent tube-worm *Riftia pachyptila*. *Mar. Biol.* **83**, 109-124.

Childress, J. J., Lee, R. W., Sanders, N. K., Felbeck, H., Oros, D. R., Toulmond, A., Desbryéres, D., Kennicutt, M. C., II and Brooks, J. (1993). Inorganic carbon uptake in hydrothermal vent tubeworms facilitated by high environmental pCO_2. *Nature* **362**, 147-149.

Cordes, E. E., Bergquist, D. C., Shea, K. and Fisher, C. R. (2003). Hydrogen sulfide demand of long-lived vestimentiferan tubeworm aggregations modifies the chemical environment at deep-sea hydrocarbon seeps. *Ecol. Lett.* **6**, 212-219.

Cordes, E. E., Arthur, M. A., Shea, K., Arvidson, R. S. and Fisher, C. R. (2005a). Modeling the mutualistic interactions between tubeworms and microbial consortia. *PLOS Biol.* **3**, e77.

Cordes, E. E., Hourdez, S., Predmore, B. L., Redding, M. L. and Fisher, C. R. (2005b). Succession of hydrocarbon seep communities associated with the long-lived foundation species *Lamellibrachia luymesi*. *Mar. Ecol. Prog. Ser.* **305**, 17-29.

Culliford, S. J., Ellory, J. C., Lang, H.-J., Englert, H., Staines, H. M. and Wilkins, R. J. (2002). Specificity of classical and putative Cl^- transport inhibitors on membrane transport pathways in human erythrocytes. *Cell. Physiol. Biochem.* **13**, 181-188.

de Cian, M.-C., Anderson, A. E., Toullec, J.-Y., Biegela, I., Caprais, J.-C., Shillito, B. and Lallier, F. H. (2003). Isolated bacteriocyte cell suspensions from the hydrothermal-vent tubeworm *Rifita pachyptila*, a potent tool for cellular physiology in a chemoautotrophic symbiosis. *Mar. Biol.* **142**, 141-151.

Fisher, C. R., Urcuyo, I. A., Simpkins, M. A. and Nix, E. R. (1997). Life in the slow lane: growth and longevity of cold-seep vestimentiferans. *Mar. Ecol.* **18**, 83-94.

Freytag, J. K., Girguis, P. R., Bergquist, D. C., Andras, J. P., Childress, J. J. and Fisher, C. R. (2001). A paradox resolved: Sulfide acquisition by roots of seep tubeworms sustains net chemoautotrophy. *Proc. Natl. Acad. Sci. USA* **98**, 13408-13413.

Gardiner, S. L. and Hourdez, S. (2003). On the occurrence of the vestimentiferan tube worm *Lamellibrachia luymesi* van der Land and Nørrevang, 1975 (Annelida: Pogonophora) in hydrocarbon seep communities in the Gulf of Mexico. *Proc. Biol. Soc. Wash.* **116**, 380-394.

Gardiner, S. L. and Jones, M. L. (1993). Vestimentifera. In *Microscopic Anatomy of Invertebrates: Vol. 12, Onychophora, Chilopoda, and Lesser Protostomata* (ed. F. W. Harrison and M. E. Rice), pp. 371-460. New York: Wiley-Liss.

Gelband, C. H., Greco, P. G. and Martens, J. R. (1996). Voltage-dependent chloride channels: invertebrates to man. *J. Exp. Zool.* **275**, 277-282.

Gerencser, G. A., Ahearn, G. A. and Cattey, M. A. (1996). Antiport-driven sulfate secretion in an invertebrate epithilium. *J. Exp. Zool.* **275**, 269-276.

Gerencser, G. A., Ahearn, G. A. and Cattey, M. A. (1999). Sulfate/bicarbonate antiport by lobster hepatopancreatic basolateral membrane vesicles. *J. Exp. Zool.* **284**, 158-167.

Girguis, P. R., Childress, J. J., Freytag, J. K., Klose, K. and Stuber, R. (2002). Effects of metabolite uptake on proton-equivalent elimination by two species of deep-sea vestimentiferan tubeworm, *Riftia pachyptila* and *Lamellibrachia luymesi*: proton elimination is a necessary adaptation to sulfide-oxidizing chemoautotrophic symbionts. *J. Exp. Biol.* **205**, 3055-3066.

Goffredi, S. K. and Childress, J. J. (2001). Activity and inhibitor sensitivity of ATPases in the hydrothermal vent tubeworm *Riftia pachyptila*: a comparative approach. *Mar. Biol.* **138**, 259-265.

Goffredi, S. K., Childress, J. J., Desauliniers, N. T., Lee, R. W., Lallier, F. H. and Hammond, D. (1997). Inorganic carbon acquisition by the hydrothermal vent tubeworm *Riftia pachyptila* depends upon high external pCO_2 and upon proton-equivalent ion transport by the worm. *J. Exp. Biol.* **200**, 883-896.

Goffredi, S. K., Childress, J. J., Lallier, F. H. and Desaulniers, N. T. (1998). How to be a perfect host: CO_2 and HS^- accumulation and H^+ elimination in the hydrothermal vent tube-worm *Riftia pachyptila*. *Cah. Biol. Mar.* **39**, 297-300.

Goffredi, S. K., Childress, J. J., Lallier, F. H. and Desaulniers, N. T. (1999). The ionic composition of the hydrothermal vent tubeworm *Riftia pachyptila*: Evidence for the elimination of SO_4^{2-} and H^+ and for a Cl^-/ HCO_3^- shift. *Physiol. Biochem. Zool.* **72**, 296-306.

Hille, B. (1992). *Ionic Channels of Excitable Membranes*. Sunderland, MA: Sinauer Associates.

Jones, M. L. (1981). *Riftia pachyptila*, new genus, new species, the Vestimentiferan tubeworm from the Galapagos Rift geothermal vents. *Proc. Biol. Soc. Wash.* **93**, 1295-1313.

Joye, S. B., Boetius, A., Orcutt, B. N., Montoya, J. P., Schulz, H. N., Erickson, M. J. and Lugo, S. K. (2004). The anaerobic oxidation of methane and sulfate reduction in sediments from Gulf of Mexico cold seeps. *Chem. Geol.* **205**, 219-238.

Julian, D., Gaill, F., Wood, E., Arp, A. and Fisher, C. (1999). Roots as a site of hydrogen sulfide uptake in the hydrocarbon seep vestimentiferan *Lamellibrachia* sp. *J. Exp. Biol.* **202**, 2245-2257.

Kennicutt, M. C., II, Burke, R. A., Jr, MacDonald, I. R., Brooks, J. M., Denoux, G. J. and Macko, S. A. (1992). Stable isotope partitioning in seep and vent organisms: chemical and ecological significance. *Chem. Geol.* **101**, 293-310.

Leustek, T. and Saito, K. (1999). Sulfate transport and assimilation in plants. *Plant Physiol.* **120**, 637-643.

Lodish, H., Berk, A., Zipursky, L. S., Matsudaira, P., Baltimore, D. and Darnell, J. (2000). *Molecular Cell Biology*. New York: WH Freeman.

MacDonald, I. R. (1998). Habitat formation at Gulf of Mexico hydrocarbon seeps. *Cah. Biol. Mar.* **39**, 337-340.

Markovich, D. (2001). Physiological roles and regulation of mammalian sulfate transporters. *Physiol. Rev.* **81**, 1500-1533.

Pflugfelder, B., Fisher, C. R. and Bright, M. (2005). The color of the trophosome: elemental sulfur distribution in the endosymbionts of *Riftia pachyptila* (Vestimentifera; Siboglinidae). *Mar. Biol.* **146**, 895-901.

Pritchard, J. B. and Renfro, J. L. (1983). Renal sulfate transport at the basolateral membrane is mediated by anion exchange. *Proc. Natl. Acad. Sci. USA* **80**, 2603-2607.

Renfro, J. L. (1999). Recent developments in teleost renal transport. *J. Exp. Zool.* **283**, 653-661.

Renfro, J. L. and Pritchard, J. B. (1983). Sulfate transport by flounder renal tubule brush border: presence of anion exchange. *Am. J. Physiol.* **244**, F488-F496.

Roberts, H. H. and Aharon, P. (1994). Hydrocarbon-derived carbonate buildups of the northern Gulf of Mexico continental slope: a review of submersible investigations. *Geo-Mar. Lett.* **14**, 135-148.

Sassen, R., MacDonald, I. R., Requejo, A. G., Guinasso, N. L., Jr, Kennicutt, M. C., II, Sweet, S. T. and Brooks, J. M. (1994). Organic geochemistry of sediments from chemosynthetic communities, Gulf of Mexico slope. *Geo-Mar. Lett.* **14**, 110-119.

Shimuzu, M. and Bradley, T. J. (1994). Concentrated excretion of sulfate by the anterior colon of the brine fly, *Ephydra hians*. *Physiol. Zool.* **67**, 54-67.

Valentine, D. L. (2002). Biogeochemistry and microbial ecology of methane oxidation in anoxic environments: a review. *Antonie Van Leeuwenhoek* **81**, 271-282.

van der Land, J. and Nørrevang, A. (1977). Structure and relationships of *Lamellibrachia* (Annelida, Vestimentifera). *K. Dan. Vidensk. Selsk. Biol. Skr.* **21**, 1-102.

Yildiz, F. H., Davies, J. P. and Grossman, A. R. (1994). Characterization of sulfate transport in *Chlamydomonas reinhardtii* during sulfur-limited and sulfur-sufficient growth. *Plant Physiol.* **104**, 981-987.