Effects of hydrogen sulfide on the feeding activity of Manila clam *Ruditapes philippinarum*

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ABSTRACT: After being exposed to anoxia for 24 h, with and without hydrogen sulfide (H_2S; 5 or 30 mg l^{-1}), the feeding activities of the Manila clam *Ruditapes philippinarum* were examined in the laboratory under normoxic conditions. The clearance rates of clams after exposure to anoxia with 30 mg l^{-1} H_2S (5.1 l h^{-1} ind.$^{-1}$) were significantly lower than in those exposed to normoxic or anoxic conditions (11.4 or 11.2 l h^{-1} ind.$^{-1}$, respectively). The phytopigment concentration of the digestive glands of clams treated with 30 mg l^{-1} H_2S under anoxic conditions (41.7 µg g^{-1}, wet weight of digestive gland) was significantly lower than that measured in other experimental treatments in this study (548.4 µg g^{-1} for normoxia, 461.9 µg g^{-1} for anoxia only, 479.8 µg g^{-1} for 5 mg l^{-1} sulfidic anoxia; p < 0.01). Histological examination of clam tissue revealed that the ciliary epithelium was damaged by a high concentration of H_2S. These findings indicate that once the clams suffered damage to their gills by exposure to H_2S, their feeding activity was impaired and they could not ingest prey, even when their environment was returned to optimal conditions.

KEY WORDS: Clearance rate · Gill · Digestive gland · Ingestion · Histology

INTRODUCTION

Bottom water in eutrophic, closed, inner bays and neritic regions often becomes anoxic during the summer stratification period, due to oxygen consumption by organic material, that has either been discharged by human activity or produced naturally. Oxygen consumption in the bottom sediment, associated with organic decomposition, causes the seabed environment to shift from an oxidizing to a reducing condition. Under reducing conditions, hydrogen sulfide (H_2S) is often produced by sulfate reduction. H_2S is harmful to aerobic organisms because it inhibits the respiratory enzyme cytochrome *c* oxidase (Smith et al. 1977). Therefore, the generation of H_2S in sediment or in the bottom layer of a water body has a strong negative effect on the survival of benthic infauna (Vismann 1991, Diaz & Rosenberg 1995). In oxygen-saturated seawater, however, H_2S has a half-life of about 20 min (Ostlund & Alexander 1963). Thus, there is not much effect of H_2S in oxygen saturated seawater.

The Manila clam *Ruditapes philippinarum* (Adams & Reeve, 1850) dominates the intertidal and upper subtidal zones of sandy tidal flats or shallow coastal areas along the eastern coast of Asia, the western coast of North America, and the northern coast of the Mediterranean; it is an important fishery resource in these regions (Chew 1989). Anoxic conditions and H_2S occur sporadically in the habitats of this clam (Yatsuka 1953, Hagita 1985, Higano 2005). Several studies have examined the combined effects of anoxia and H_2S on the survival or metabolic rates of various bivalves (Hagita 1985, Shumway et al. 1993, Oeschger & Pedersen 1994, Nakamura et al. 1997, Laudien et al. 2002, Le Moullac et al. 2008). Accord-

ing to these reports, bivalves that were cultured under anoxic conditions with H_2S in the water for several consecutive days were much less likely to survive than those cultured under anoxic conditions without H_2S. Within the biotope of intertidal bivalves, however, the anoxic sulfidic condition would not continue for several days, because the substratum is exposed to air, and oxygen penetrates into the sediment during every tidal cycle. Furthermore, some intertidal bivalves can tolerate H_2S to some extent by oxidizing this poisonous compound into nontoxic compounds, mainly thiosulfate (Jahn et al. 1997, Jahn & Theede 1997, Laudien et al. 2002). Therefore, it is unlikely that H_2S would be a direct cause of mortality of Manila clams in their natural environment. Vaquer-Sunyer & Duarte (2010) suggested that the fact that intertidal bivalves may remain alive after exposure to anoxia or sulfide does not necessarily mean they will survive long-term, as they may have been compromised enough to die subsequently of other causes.

To date, no studies have examined the physiological sequelae or histological lesions of bivalves following exposure to H_2S. The objectives of the present study were to investigate the effects of H_2S exposure on the feeding behavior of Manila clams and to examine whether the clams' gill tissue is damaged by H_2S.

MATERIALS AND METHODS

Pre-experimental farming condition

The Manila clams used for the experiments had a shell length of 34.13 ± 1.79 mm (mean ± SD, n = 46) and were collected in Konagai-cho, Nagasaki, Japan (32° 55′ 00″ N, 130° 10′ 10″ E). The clams were farmed in an indoor tank at 23°C and a salinity of 33. They were fed a mixture of the diatom *Chaetoceros gracilis* (Tasaki Institute for Marine Biological Research) and Shellfish Micron M1 (Nosan), a commercial feed. Prior to the experiment, in order to clean their gut contents, the clams were kept for 5 d in a breeding tank through which aerated, algae-free seawater (23°C, salinity 33) flowed.

Experimental design

To examine the impact of H_2S under anoxic conditions (sulfidic anoxia), the clams were exposed to 2 different H_2S concentrations. The higher concentration (30 mg l^{-1}) was established based on H_2S measurements by Sakai et al. (2004) in surface sediment

pore water (maximum concentration ~37 mg l^{-1}) in Lake Nakaumi, Japan, where Manila clams live. The lower concentration (5 mg l^{-1}) was established based on the field investigation of Hagita (1985).

The experiment consisted of a series of 3 procedures, described below. The entire series was performed twice.

Procedure 1: Anoxic incubation with and without H_2S

Clams were exposed to anoxic conditions for 24 h, with and without H_2S, using the equipment illustrated in Fig. 1. Five clams were placed into an airtight separable flask (As One), and the cover was held in place tightly with 3 clamps. The flask was filled with 1.5 l nitrogen-bubbled oxygen-free filtered seawater (23°C, salinity 33, pH adjusted to 7.5), and the upper half of the flask was sealed with a stopper; no air space was left in the separable flask. Dissolved oxygen (DO) of the seawater was monitored by an Oxi 340i DO sensor (WTW) during the period of nitrogen bubbling. DO concentration remained below 0.4 mg l^{-1}.

For anoxic exposure experiments with H_2S, clams were incubated as described above. To introduce H_2S, a $Na_2S \cdot 9H_2O$ solution was prepared and then immediately added to the nitrogen-bubbled oxygen-free filtered seawater to a final concentration of either 5 or 30 mg l^{-1} (pH 7.5). To prepare this solution, crystals of $Na_2S \cdot 9H_2O$ were weighed into a 30 ml
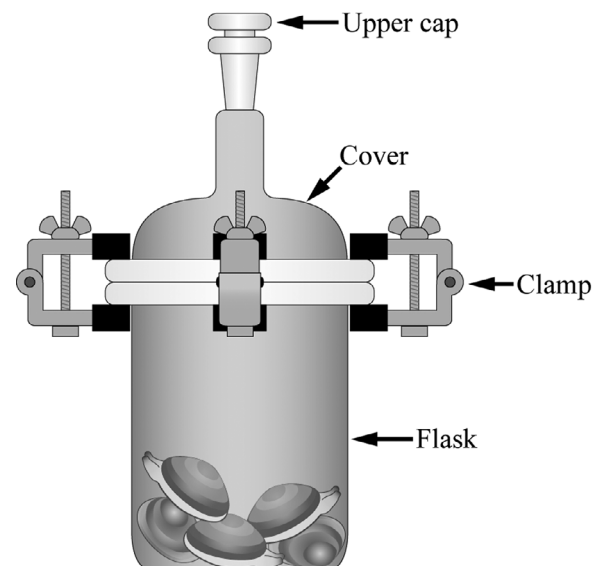

Fig. 1. Separable-flask exposure apparatus

glass syringe (Tsubasa Industry), and 10 to 20 ml of oxygen-free filtered seawater were slowly drawn into the syringe without aeration. 4N HCl was injected through an inlet of the syringe to adjust the pH of the water in the syringe to 7.5. The H_2S solution was then injected into the seawater of each flask without aeration, the separable flask was filled with oxygen-free seawater to the maximum flask volume, and the upper cap was sealed. To confirm the concentration of H_2S in the separable flasks, we conducted a preliminary experiment to analyze the concentration using the methylene blue method (Cline 1969). In this paper, the term sulfide refers to all forms of H_2S (H_2S, HS^-, and S^{2-}).

As a control for the anoxic incubation with and without H_2S, we also incubated clams under normoxic conditions using the same equipment (Fig. 1). Five Manila clams were incubated in a separable flask containing 1.5 l of aerated filtered seawater (23°C, salinity 33, pH adjusted to 7.5) without the upper cap in place. Additionally, the seawater in the flask was gently aerated to prevent a reduction in DO concentration.

Procedure 2: Measurement of clearance rate

In order to examine the effect of sulfide, the measurement of the clams' clearance rate was made under laboratory normoxic conditions according to the method outlined by Nagasoe et al. (2011). The clams used for this examination were the individuals that had been treated in Procedure 1. Six polypropylene beakers were prepared for each treated group (normoxic incubation, anoxic incubation without H_2S, and anoxic incubation with 5 or 30 mg l^{-1} H_2S). Two liters of filtered seawater were added to each polypropylene beaker, and one clam each was placed into 5 beakers (hereinafter called 'assay beakers'). The sixth beaker ('control beaker') was left without a clam for the purpose of calculating a correction coefficient (see Eq. 1). The clams were stored under dim light at 23 ± 1°C, and the temperature was maintained by an electrically heated water bath. After the clams were placed in the beakers, they were left undisturbed for 30 min. A culture of the diatom *Chaetoceros gracilis* was added to all 6 beakers to a final concentration in each beaker of ~2.5 × 10⁴ cells ml^{-1} (prior to this inoculation, a volume of seawater equal to the algae culture's inoculum volume was drained from each beaker). The seawater in the beakers was gently aerated to ensure a homogeneous distribution of phytoplankton. The cell concen-
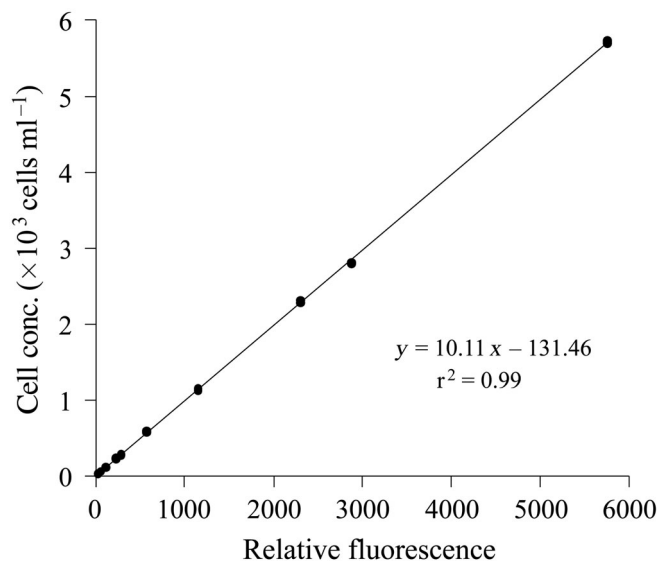

Fig. 2. Correlation between cell concentration and *in vivo* fluorescence of diatom *Chaetoceros gracilis*

tration of phytoplankton in each beaker was measured every hour for 4 h.

The abundance of diatom cells was determined by measuring *in vivo* chl *a* fluorescence with a Model 8000-010 fluorometer (Turner Designs). Prior to the experiment's initiation, the correlation between cell concentration and *in vivo* fluorescence units of microalgae was examined (Fig. 2). Although the diatom concentration in the control beaker did not increase during these experiments (as confirmed by direct count under a light microscope), the *in vivo* chl *a* fluorescence per unit water volume increased slightly with time. This is a result of an increase in the *in vivo* chl *a* per cell (Fig. 3). We therefore calculated a correction coefficient to keep the *in vivo* chl *a* fluorescence of the control beaker constant:

$$\text{Correction coefficient} = \frac{F_0}{F_t} \qquad (1)$$

where F_0 is the *in vivo* chl *a* fluorescence of the diatoms in the control beaker at the time of addition of inoculum and F_t is the *in vivo* chl *a* fluorescence of the diatoms after *t* hours. We corrected the cell concentration values of the assay beakers by multiplying them by this correction coefficient.

The rate of phytoplankton clearance by each clam (C; l h^{-1} $ind.^{-1}$) was calculated using the ln-transformed diatom cell concentration data for the 4 h measurement period according to the method of Coughlan (1969):

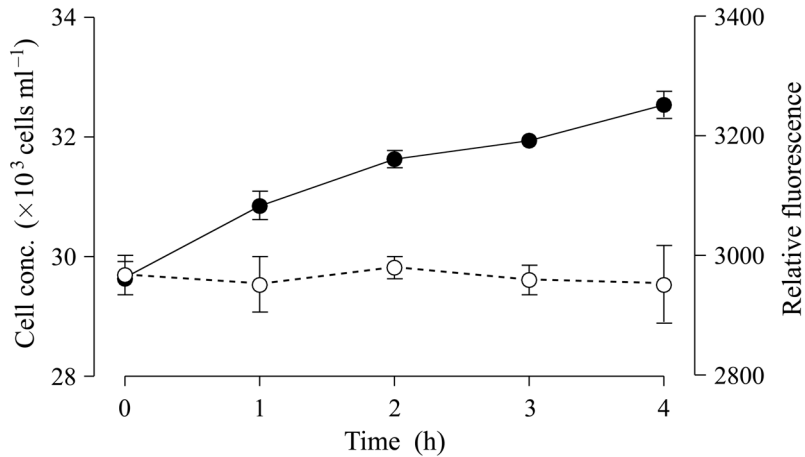$$C = \frac{V}{n \times t} \times \ln \frac{N_0}{N_t} \qquad (2)$$

Fig. 3. Time-dependent changes in diatom cell concentration (O, left axis) and *in vivo* chl *a* fluorescence per ml (●, right axis) in control beaker. Error bars indicate range of measured values

where N_0 is the cell concentration (cells ml^{-1}) at the time of addition of inoculum, N_t is the cell concentration (cells ml^{-1}) after t hours, V is the volume (l) of water in the beaker, and n is the number of clams. In this experiment, the values of these variables were exactly $t = 4$, $V = 2$, and $n = 1$.

Procedure 3: Analysis of phytopigment in digestive glands, and histological examination of tissue

After measuring clearance rate, the clams were immediately dissected and a piece of digestive gland (about 0.05 g) was isolated from each clam. The analysis of phytopigments (chl *a* + phaeophytin) in the digestive glands, carried out immediately after sample isolation, was performed according to the method of Numaguchi (2001). Each piece of wet digestive gland tissue was weighed, soaked in 10 ml of 90% acetone in the dark at 4°C for 24 h, and then centrifuged at 2300 × *g* for 20 min to extract the pigment. After the addition of 50 µl of 4N HCl, ~5 ml of the supernatant was measured with a spectrophotometer at wavelengths of 665 and 750 nm to determine its phytopigment content. The phytopigment concentration (µg g^{-1}) was determined using Eq. (3), which was modified from an equation developed by Strickland & Parsons (1968):

$$\text{Phytopigment conc.} = \frac{2.67 \times (1.7E_a - E_a) \times v}{M \times l} \quad (3)$$

where E_a is the absorbance difference between the values at 665 and 750 nm after the addition of 50 µl of

4N HCl to the supernatant, v (ml) is the volume of acetone used for the pigment extraction, M (g) is the wet weight of the digestive gland, and l (cm) is the width of the quartz spectrophotometer cell.

We also examined whether anoxia, with or without H_2S, caused damage to clam tissue. Three clams were randomly chosen from the 5 in each treatment 'assay beaker' (see Procedure 2) in one run (total 6 clams were used for each treatment group in 2 runs). One 5 mm cross-section was sampled from the middle region of the gill in each clam, fixed in a 10% mixture of formalin in seawater, and then dehydrated and embedded in paraffin. Three 5 µm tissue sections obtained from different areas of each cross-section (one tissue section at 100 µm from each end and one from the middle of the block) were stained with Mayer's haematoxylin and eosin. The sections were examined under a light microscope, with each section rated using a scoring system based on extent of observed damage: zero = no damage, indicating normal gill tissue (Fig. 4A); 1 = slightly damaged, with infiltration of hemocytes in connective tissue (Fig. 4B); 2 = severely damaged, with cilia detached from filament epithelial cells (Fig. 4C); 3 = diapedesis, passage of hemocytes through the damaged epithelial cells of the gill filament (Fig. 4D). Each biopsy specimen was examined by 2 pathologists (Y. Maeno & T. Yurimoto). The gill damage on each clam was evaluated as the sum of the scores on 3 tissue sections.

Statistical analysis

The clearance-rate and phytopigment content values in digestive glands for each experimental treatment group were averaged for each 10 clams (total in 2 runs), and are expressed as mean ± SD. All data sets were tested for normal distribution and homogeneity of variances prior to analysis. Because data sets for clearance rates showed normal distributions and homogeneity of variances, post hoc analysis was performed with Tukey's HSD test. Because the data sets for phytopigment content of digestive glands were not normally distributed, the nonparametric Kruskal-Wallis test was used for that analysis. Subsequently, the effects of the treatments were also tested with Mann-Whitney U-tests with a Bonferroni correction.
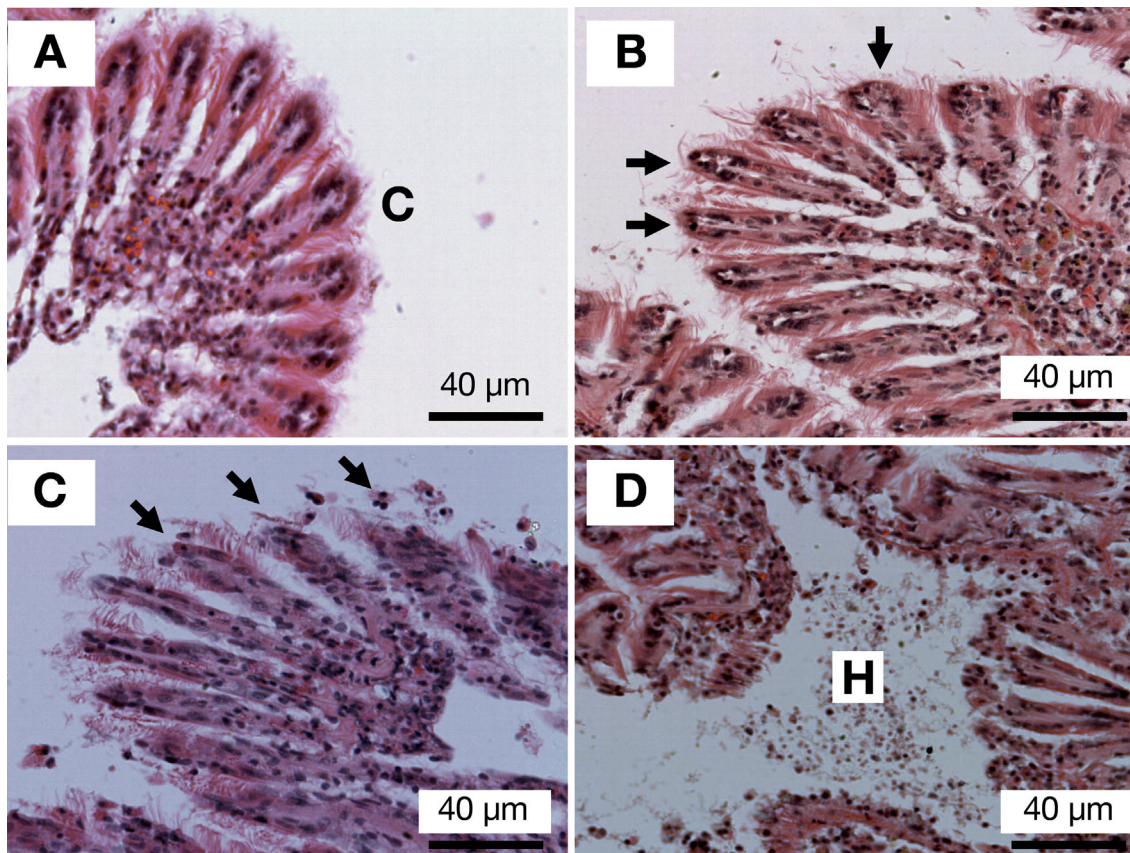
Fig. 4. *Ruditapes philippinarum*. Light-microscope images of gill tissue exposed to (A) normoxia and (B,C,D) anoxia with 30 mg l^{-1} H$_2$S. (A) Normal gill tissue; (B) slightly damaged tissue, with infiltration of hemocytes in connective tissue; (C) severely damaged tissue, with cilia detached from epithelial cells of the filament; (D) diapedesis, i.e. passage of hemocytes through damaged epithelial cells of gill filament. Arrows point to exfoliated cilia from epithelial cells of filament. C: cilia; H: hemocyte

The histological scores for each experimental treatment group were the averages of scores for each of 6 clams (total in 2 runs), and are expressed as mean ± SE. The Kruskal-Wallis test for significance was performed for the 4 groups (normoxia, anoxia only, 5 and 30 mg l^{-1} sulfidic anoxia). If a significant difference was seen, a Mann-Whitney *U*-test was performed between the normoxic group and the other groups. Statistical analyses were performed using SPSS 10.0 for Windows, with significance set at the p < 0.05 level.

RESULTS

Anoxic incubation with and without H$_2$S

In the first experiment (Procedure 1), the mean initial concentrations of H$_2$S in each separable flask were 0.0 ± 0.0 mg l^{-1} (mean ± SD, n = 3, normoxic conditions), 0.0 ± 0.0 mg l^{-1} (anoxia without H$_2$S), 5.1 ±

0.3 mg l^{-1} (H$_2$S concentration of 5 mg l^{-1}), and 27.7 ± 2.5 mg l^{-1} (H$_2$S concentration of 30 mg l^{-1}).

No mortality occurred in the clams exposed to anoxia with or without H$_2$S for 24 h, even at the high H$_2$S concentration (30 mg l^{-1}). Although we observed clams reaction only a few times during the 24 h exposure period, all clams in H$_2$S solutions, whether at 5 or 30 mg l^{-1}, extended their siphons into the water column. On the other hand, all clams under normoxia nearly closed their shells, and only slightly extended their siphons through the barely parted shell halves. Some clams under only anoxia extended their siphons at a length intermediate between those under normoxia and those under H$_2$S anoxic conditions. Other clams under anoxia alone reacted similarly to those under normoxia. Moreover, at the end of the 24 h exposure, all clams picked out of sulfidic water were unable to withdraw their siphons for some time. In contrast, the clams exposed to normoxia or to anoxia alone withdrew their siphons immediately, and closed their shell.
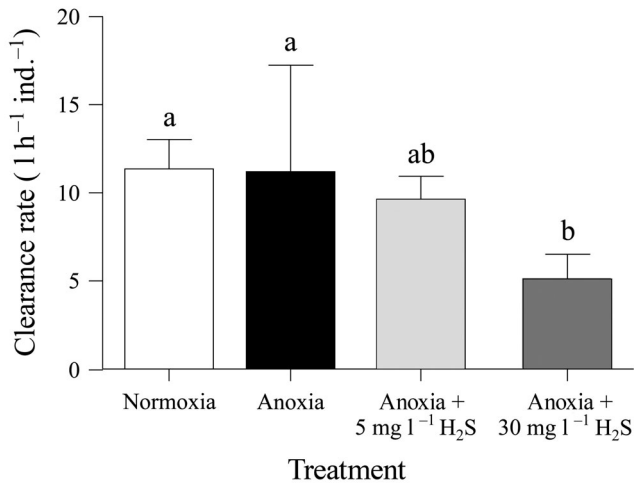
Fig. 5. *Ruditapes philippinarum*. Clearance rates of clams exposed to various experimental conditions. Data represent the mean ± SD (n = 10). Means with same letter are not significantly different (p > 0.05, Tukey's HSD test)

Clearance rates

As shown in Fig. 5, there was no significant difference in clearance rates between the clams treated with 5 or 30 mg l^{-1} H$_2$S (Tukey's HSD test, p = 0.189), but the clearance rate for clams treated with 30 mg l^{-1} H$_2$S was significantly lower than those of clams exposed to normoxic (p = 0.038) or only anoxic conditions (p = 0.045; Fig. 5).

At the end of the diatom exposure experiment, feces and pseudofeces were left at the bottom of each beaker. In the clams exposed either to normoxic conditions, anoxia only, or 5 mg l^{-1} sulfidic anoxia, feces had a sturdy and cylindrical appearance (Fig. 6A). Upon observation under a light microscope, the feces showed a dense composition (Fig. 6A). Pseudofeces of these clams had a fragile and amorphous appearance; the diatom cells were wrapped in mucus and each individual cell could be identified easily (Fig. 6B). In contrast, the clams exposed to 30 mg l^{-1} sulfidic anoxia ejected large blobs, distinct, both in form and degree of frailty, from the feces and pseudofeces of clams in the other treated groups; the blobs appeared to be sturdy and amorphous (Fig. 6C). The diatom cells found both in the large blobs and in the pseudofeces could be identified in each cell (Fig. 6C).

Analysis of phytopigments in the digestive gland

As shown in Fig. 7, phytopigment content of clams treated with 30 mg l^{-1} sulfidic anoxia was similar to that of food-deprived clams (initial control; Mann-

Whitney *U*-tests with Bonferroni correction, p = 0.448), but was significantly lower than that of the other experimental groups (p < 0.01). In addition, the phytopigment content of clams exposed to only anoxic conditions or to 5 mg l^{-1} sulfidic anoxia was as high as that of clams under normoxic conditions (p = 0.496 and p = 0.650, respectively; Fig. 7).

Histological examination of gills

No lesions were seen in clams exposed to normoxic conditions (Fig. 4A). In 1 and 2 individuals out of the groups of 6 clams exposed to either anoxia only or low sulfidic anoxia (5 mg l^{-1}), respectively, slight lesions were observed in some areas of the gill tissue (Fig. 4B). No lesions were seen in any other clams of those experimental groups. Subsequently, no significant difference was observed between the histological score in clams treated under normoxic condition and the score in clams exposed to anoxia (Mann-Whitney *U*-test, p = 0.699) or low sulfidic anoxia (p = 0.394; Table 1). In the clams exposed to anoxia with a high concentration of H$_2$S (30 mg l^{-1}), slight damage was observed in almost all of the gill tissue sections (Fig. 4B). Moreover, cilia detached from the epithelium of the gill filament (severe damage; Fig. 4C) and the passage of the epithelial cells of the gill filament (diapedesis; Fig. 4D) were observed only in the clams treated under high sulfidic anoxia (30 mg l^{-1}). Among 6 clams, 2 were suffering severe damage and 3 diapedesis. Consequently, the histological score in the high-sulfidic anoxia group (30 mg l^{-1}) was significantly higher than in the normoxia group (p = 0.015; Table 1).

DISCUSSION

The tendency of some infaunal bivalves in sulfidic water to extend their siphons upward into the water column has been reported in both field (Jørgensen 1980) and laboratory studies (Rosenberg et al. 1991, Shumway et al. 1993, Oeschger & Pedersen 1994, Laudien et al. 2002). Laudien et al. (2002) suggested that this extrusion of siphons into sulfidic water might be coupled with higher ventilation activity, but served mainly to monitor the water for better conditions. Oeschger & Pedersen (1994) suggested that the siphons might contain a diffusive barrier against sulfide. We found that the manner of extrusion of siphons differed between the clams exposed to anoxia with and without sulfide. These observations suggest
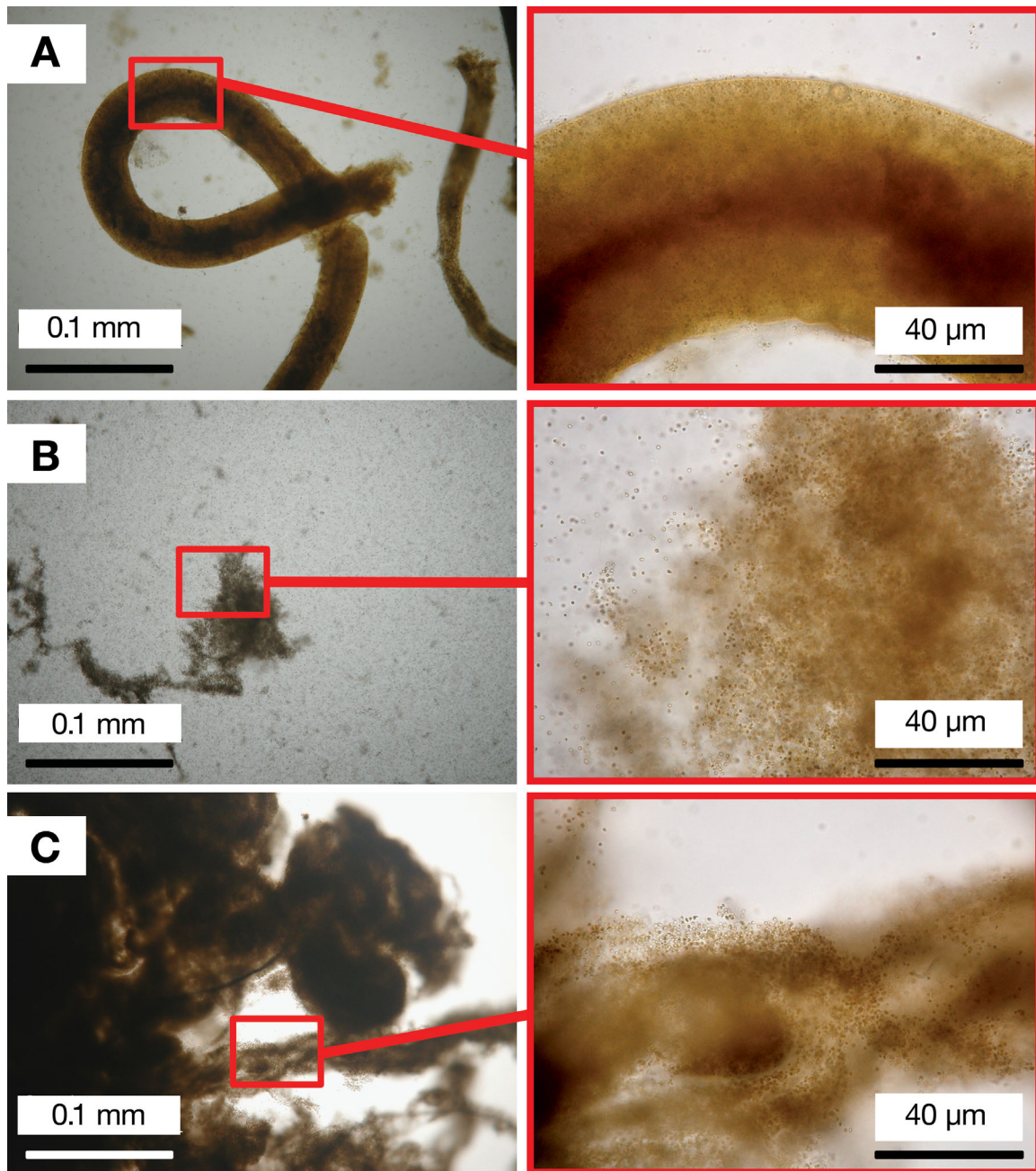
Fig. 6. *Ruditapes philippinarum*. Light-microscope images of fecal substances collected at the end of diatom exposure experiment. (A) Typical feces and (B) pseudofeces of clams exposed to normoxic conditions, anoxia only, or 5 mg l^{-1} sulfidic anoxia. (C) Typical material rejected by clams exposed to 30 mg l^{-1} sulfidic anoxia

that clams consciously extrude their siphons to obtain water of higher DO concentration. In the case of exposure to sulfidic anoxia, however, the siphons of clams involuntarily extended upward into the water column, from which we infer that H$_2$S may cause muscle relaxation. Therefore, clams (with extended siphons) removed from sulfidic water proved unable to withdraw their siphons immediately. Further studies are needed to understand why bivalves extrude their siphons in the presence of sulfide.

Marine invertebrates inhabiting H$_2$S-rich marine sediments have acquired a variety of biochemical adaptations to detoxify this poisonous compound (Vismann 1991, Bagarinao 1992, Völkel & Grieshaber
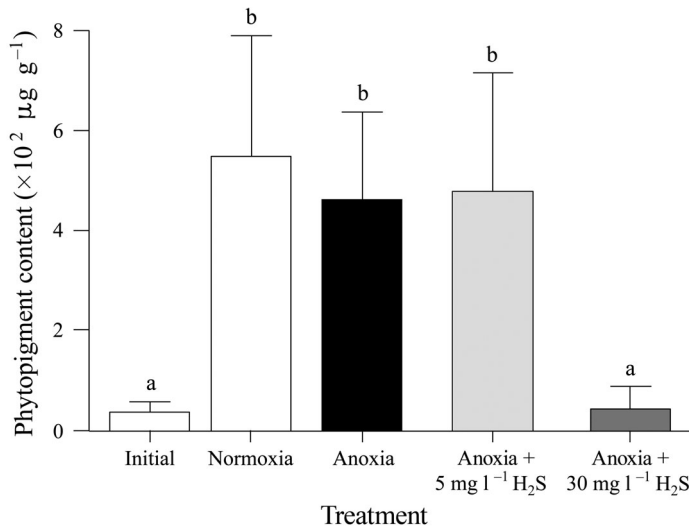
Fig. 7. *Ruditapes philippinarum*. Content of phytopigment (chl *a* + phaeophytin) in digestive glands of clams exposed to various experimental conditions. Initial level is the concentration of phytopigment in digestive glands before exposure to diatoms. Data represent mean ± SD (n = 10, except for initial control n = 6). Means with same letter are not significantly different (p > 0.01, Mann-Whitney *U*-test with Bonferroni correction)

Table 1. Comparison of histological lesion scores between the normoxic group and the other treatment groups. *p < 0.05

Experimental treatment group	Histological score (n = 6) Mean ± SE	p
Normoxia	0 ± 0	
Anoxia without H_2S	0.17 ± 0.17	0.699
Anoxia with 5 mg l^{-1} H_2S	0.67 ± 0.42	0.394
Anoxia with 30 mg l^{-1} H_2S	3.33 ± 1.28	0.015*

1995, Grieshaber & Völkel 1998). In marine bivalves, sulfide is metabolized to less toxic or non-toxic compounds through oxidizing cell tissue activity (Cary et al. 1989, O'Brien & Vetter 1990). Thus, bivalves could tolerate H_2S if their body tissue were oxygenated. Because gill tissue is in intimate contact with ambient water, gill-tissue oxygen will be consumed for detoxification in H_2S-containing water. Our observations clearly indicated that gill tissue is susceptible to the damaging effects of H_2S.

Gills of bivalves act not only as respiratory organs but also as suspension-feeding organs. Suspended particles on the surface of bivalve gills are transferred to the mouth by ciliary movement. Gainey (2007) compiled previous results on particle capture and transport by gills and found that both the coordi-

nated activity of cilia (3 groups of them) and gill geometry are absolutely essential to effective particle transport. Subtle changes in the structure of gill tissues can therefore lead to feeding dysfunction (Ward et al. 1993). In our study, clams exhibited difficulty in feeding when their gill tissues were damaged only at a high concentration of H_2S (30 mg l^{-1}; Fig. 4C,D). Because the exposure of clams to H_2S in our study was effected by a batch-culture technique, the initial concentration of H_2S likely decreased gradually during the 24 h exposure period. This may have been due, e.g. to the clams' detoxification of H_2S. However, we did not analyze the time-dependent decline in the concentration of H_2S. If the low concentration of H_2S (5 mg l^{-1}) had been maintained throughout the experimental period, the gill tissue might have been damaged more seriously than our observations indicate, and the clearance rate would likely have declined with the degree of gill damage. Alternatively, serious damage to gill tissue may be produced by the accumulation of slight injuries due to periodic exposure to low concentrations of H_2S. In either case, further studies should undertake to examine the impact of low concentrations of H_2S on the physiological processes and activities of bivalves, including respiration and feeding.

Mucus plays a key role in all aspects of particle processing on the gill surface (Beninger et al. 1991, Beninger et al. 1993, 1997a,b, Beninger & Le Pennec 1993, Beninger & Dufour 1996, Beninger & St-Jean 1997a,b, Wotton 2004). Mucus consists of polysaccharide units with associated protein components (muco-polysaccharides; MPS). The MPS can be divided into 3 classes according to degree of acidity and viscosity: (1) acid MPS (AMPS) and acid-dominant MPS (ADMPS), both exhibiting high viscosity; (2) neutral MPS (NMPS), exhibiting low viscosity; and (3) mixed MPS (MMPS), containing roughly equal proportions of AMPS and NMPS, and exhibiting relatively low viscosity (Beninger & St-Jean 1997b). In addition, these various classes of MPS play particular roles in gill-surface particle processing. High-viscosity AMPS and ADMPS act to reject pseudofeces; lower-viscosity MMPS is involved in transport of particles destined for ingestion; and low-viscosity NMPS dilutes AMPS and ADMPS to reduce their viscosity (Beninger and St-Jean 1997b). The properties and components of mucus are therefore important for the feeding activity of bivalves. Thus clams exposed to a high concentration of H_2S (30 mg l^{-1}) may secrete mucus with altered components resulting from direct damage to gill tissue, and this in turn may disrupt particulate transport on the gill surface. Although we

did not examine the alteration of gill mucus components, this hypothesis is supported by the observation that clams exposed to high sulfidic anoxia ejected fecal substances that were different from normal feces or pseudofeces (Fig. 6).

Among clams exposed to 30 mg l^{-1} sulfidic anoxia, the diatom cell concentration decreased (through filtration for 4 h) from 28 257 ± 469 cells ml^{-1} (mean ± SE, n = 10) to 14 948 ± 2964 cells ml^{-1}. However, the amount of phytopigment in the digestive glands of these clams (41.7 µg g^{-1}) was similar to that in food-deprived clams (37.7 µg g^{-1}, p = 0.448; Fig. 7). Thus diatoms that disappeared from the water were not ingested, but were ejected as sturdy and amorphous large blobs. This finding suggests that clams damaged by H$_2$S would be more likely to die of malnutrition.

This is the first study we know of to elucidate the physiological sequelae and tissue damage of bivalves caused by exposure to H$_2$S. Our findings indicate that the Manila clam *Ruditapes philippinarum* is not killed directly by short-term exposure to H$_2$S, but that the gill tissue is damaged by exposure to H$_2$S, and the clams are therefore incapable of feeding normally. Although the environment may return to optimal conditions, such clams would starve or be gradually debilitated as a result of the sulfide-caused damage to their gills. Consequently, debilitated clams would be more susceptible to disease and death. These impacts of H$_2$S may be related to the mortality of bivalves in the field.

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