Influence of incubation conditions on the anoxic survival of marine bivalves. Static and semi-static incubations

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ABSTRACT: In a comparative study of 4 bivalve species we show that the apparent widely different tolerances in survival time observed in a closed system filled with N_2-gassed seawater is mainly due to the experimental conditions. Both a high dose of cadmium and the antibiotic chloramphenicol increase survival time 2- to 4-fold. Without precautions for bacterial growth, the survival time of the most tolerant species, *Scapharca inaequivalvis*, is about 4 times longer than that of the most sensitive species, *Venus gallina*. The other bivalves *Tapes philippinarum* and *Mytilus galloprovincialis*, have intermediate survival times. When proliferation of bacteria is prevented by chloramphenicol, the survival time of *S. inaequivalvis* remains the highest; however under these conditions the other 3 species show similar survival times. Bacteria cause a drop in pH of the incubation medium and the accumulation of sulphide. *S. inaequivalvis* exhibits a significant decrease in survival time in the presence of exogenous sulphide. The antibiotic postpones the accumulation of sulphide, but a significant population of sulphate-reducing bacteria is able to survive in its presence. In the case of *V. gallina* and *M. galloprovincialis*, exogenous sulphide (up to 400 µM) does not affect the improved survival time due to the antibiotic. This holds also for a decrease in pH. Bacterial toxins other than sulphide or direct action on the integrity of the organism must be responsible for the adverse effects on survival. The responsible micro-organisms are associated with the bivalves, and anoxia induces their proliferation. For this reason, accumulation of bacterial noxious products cannot be avoided by frequent renewal of the medium.

KEY WORDS: Benthic macrofauna · Bivalves · Hydrogen sulphide · Anaerobiosis · Cadmium · Chloramphenicol · Anoxic resistance

INTRODUCTION

Several studies have shown that exposure of bivalves to contaminants dissolved or suspended in seawater reduce their survival in air or in anoxic seawater. Anoxic survival is used in monitoring programmes to assess environmental toxicity (Eertman & de Zwaan 1994). However, studies on the uptake of the heavy metals Cd, Cu and Hg by bivalves and the effect of toxin accumulation on anaerobic energy metabolism and anoxic survival obtained unexpected results (Weber 1990, Weber et al. 1990, 1992, Cortesi et al. 1992, de Zwaan et al. 1995). We exposed the bivalves in well-aerated seawater to metal concentrations at which mortality within 1 mo was not (or only slightly) higher than in untreated control individuals. At relatively low doses of Cd and Hg, we observed the expected negative effects on anoxic survival time.

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However, when bivalves were introduced to anoxic seawater after exposure to relatively high doses of these metals, their survival times appeared to be substantially longer than that of controls. This was also the case when untreated bivalves were exposed to high concentrations of heavy metals during the anoxic incubation. Shell-valve blackening was observed in the controls, but was virtually absent in the presence of these toxins. Apparently the relatively high doses of the metals affected bacteria (causing this blackening) more than the metabolically affected bivalves. This 'bacteriostatic' effect was not only able to reverse the toxic effect of the metals, but also benefited the bivalves under anoxic stress. We realised that the median survival times estimated in static anoxic systems might underestimate the potential of these bivalves to survive anoxic conditions.

In the Adriatic Sea, populations of 2 bivalve species of great commercial importance, the 'vongole' *Venus gallina* and the blue mussel *Mytilus galloprovincialis*, are sharply decreasing, whereas populations of the recently introduced blood clam *Scapharca inaequivalvis*, a native of the Indo-Pacific region, are rapidly increasing and out-competing the 2 native species (Ghisotti & Rinaldi 1976). In order to compensate for the demise of the 2 native species, fish farms have imported the Japanese littleneck *Tapes philippinarum*. Over the last 10 yr we have been carrying out an extensive analysis of the physiological and biochemical responses to low oxygen exposure by these 4 species (Brooks et al. 1991, de Zwaan et al. 1991, Thillart et al. 1992). We have concluded that the high tolerance of *S. inaequivalvis* against anoxia explains, in part, the success of this species over the others. However, we derived our survival data from individuals sealed in closed jars, whereby bacterial interference may have led to misleading results. We therefore decided to re-investigate the anoxic survival times of these species, taking precautions against bacterial growth.

In a closed incubation system without water renewal, frequent handling of the bivalves is minimal, but with long survival times, changes in the incubation medium may occur which adversely affect anoxic survival (Felder 1979, Llansó & Diaz 1994). Several euryoxic invertebrates excrete metabolic compounds (e.g. volatile fatty acids, ethanol), others expel their gut contents when subjected to anaerobic conditions (de Zwaan & Skjoldal 1979). Fermentation products as well as excrements may provide substrates for the development of micro-organisms. Blackening of the body wall of worms and shells of bivalves kept in anoxic water points to the generation of sulphide, indicating microbial activity (Oeschger & Theede 1988). A negative effect of exogenous sulphide on anoxic survival time has been observed for a large number of

invertebrates, whereas others appear to be insensitive (de Zwaan & Eertman 1996, Gamenick et al. 1996, Jahn & Theede 1997). Unfortunately, spontaneous generation of sulphide in control individuals that may mask negative effects in the sulphide-exposed groups has, in general, not been considered.

The same holds for many studies dealing with effects of sulphide on metabolism. Artefacts in metabolic studies caused by proliferation of bacteria in anoxic medium have been reported. Frank (1983) observed that addition of antibiotics prevented the decomposition of ethanol and acetate by micro-organisms excreted by *Chironomus plumosus* larvae. Oeschger (1990) assumed that the low values for propionate in the incubation medium after long-term anaerobiosis of the bivalve *Astarte borealis* were a result of bacterial degradation. De Zwaan et al. (1982) detected large quantities of ammonia and acetate in *in vitro* incubation of adductor muscle of the blue mussel *Mytilus edulis* which was not observed *in vivo* incubation. Hummel et al. (1989) observed high acetic acid levels in the extrapallial water of emersed mussels and cockles at a time when mortality accelerated, which could only partly be accounted for by the amount of glycogen metabolised.

In order to gain better insight into how microbial activity may influence survival times, we examined for 4 bivalve species the effect of a high dose of cadmium and of the addition of the antibiotic chloramphenicol upon changing from aerated to anoxic seawater. We focussed on the role that acidification and sulphide generation, both indicators of microbial proliferation, may play on survival, and whether these effects could be counteracted by frequent renewal of the incubation medium.

MATERIALS AND METHODS

Individuals. Infaunal *Scapharca inaequivalvis* and *Venus gallina* were dredged from ca 10 m depth a few miles offshore near Cesenatico in the Adriatic Sea, Italy. Unless stated otherwise, the sediment at the collection side was sandy. Infaunal *Tapes philippinarum* and off-bottom, rope-cultured *Mytilus galloprovincialis* were obtained from a fish farm at Goro (province of Ferrara). The bivalves were placed in open baskets suspended in 3000 l polyester tanks of sand-filtered seawater at the Institute of Cesenatico. The water was well aerated and maintained at ambient seawater temperature by equilibration with the air-conditioned aquarium room. The seawater was pumped in from an inlet about 300 m offshore into a large concrete basin for sedimentation prior to filtering. Every second day, about 20% of the water was exchanged. Unless stated

otherwise, bivalves were used in the experiments 1 wk after collection. Anoxic survival was always determined at ambient seawater temperature at the start of the experiment. Unless stated otherwise, illumination was according to the ambient daily cycle.

Protocol of experiments. Anoxic seawater was prepared by vigorously bubbling natural sand-filtered seawater for about 2 h with N_2 directly into the incubation flasks or into reservoirs. The latter method was applied in the case of small incubation flasks and when compounds (sulphide, chloramphenicol, Tris) had to be added. From the reservoirs, the flasks were filled while being flushed with N_2. Chloramphenicol, $CdCl_2$ (source of cadmium) and Tris were added as dry powder, and Na_2S (the source of sulphide) from a stock solution of 200 mmol l^{-1}. The stock solution was made by adding washed crystals of $Na_2S \times 9H_2O$ to N_2-saturated distilled water. After additions, the pH was adjusted with HCl to match the values of the control anoxic seawater. The flasks were tightly closed with glass stoppers. The oxygen concentration after filling was <0.15 mg l^{-1} (Winkler method). Salinity was 32 to 34 psu. No food was added. Except for 1 experiment (later subsection 'Chloramphenicol and Tris') the experimental bivalves covered part of the bottom of the flasks in 1 layer part. The biomass volume with closed shell valves was 19.55 ± 1.72, 2.43 ± 0.22, 5.53 ± 0.28 and 15.16 ± 1.68 ml for *Scapharca inaequivalvis*, *Venus gallina*, *Tapes philippinarum* and *Mytilus galloprovincialis*, respectively. Mortality was assessed by failure of constriction upon touching the mantle edge.

Throughout this paper, the terms sulphide and H_2S refer to total sulphide, and the term Tris to the buffer Tris (hydroxymethyl)-aminomethane.

Cadmium. *Scapharca inaequivalvis* was either collected from muddy sediment (October, 17°C) or sandy sediment (November, 11°C). *Venus gallina* was collected from sandy sediment (end of September, 18°C). Upon arrival, some of the *S. inaequivalvis* collected from the sandy sediment were exposed for 2 wk to well-aerated seawater containing 100 µg Cd l^{-1} at 11°C, and anoxic survival was subsequently estimated. The remaining *S. inaequivalvis* and *V. gallina* were kept in the aquarium for 2 wk prior to experimentation. *S. inaequivalvis* and *V. gallina* were then subdivided into an untreated control group and a group subjected to a high dose of cadmium (500 µg Cd l^{-1}) dissolved in anoxic seawater. *S. inaequivalvis* was incubated anoxically at 11°C and *V. gallina at* 18°C. Two wk after collection, *S. inaequivalvis* from the muddy sediment were treated in a similar way at 17°C. In all cases, anoxic survival was determined for 50 individuals incubated in 4 l (*S. inaequivalvis*) or 2 l (*V. gallina*) flasks, each containing 16 to 17 individuals. The incubation medium was changed every day, and mortality was checked. Dead individuals were removed.

Changes in incubation water. Fifty specimens of *Venus gallina* were put in 2 l flasks filled with anoxic seawater at 18°C. Samples were taken daily to measure the concentration of ammonia, sulphide and the pH of the incubation water. The incubation water was not changed during the experiment. Dead individuals were removed from the flasks and registered for estimation of median survival time.

Chloramphenicol and sulphide. The effect of sulphide on the anoxic resistance of *Scapharca inaequivalvis* and *Venus gallina* was examined at 18°C by addition of 0.2 mmol l^{-1} H_2S and 3 mg l^{-1} chloramphenicol to the anoxic water. The controls contained only 3 mg l^{-1} chloramphenicol. Other details were as described for the cadmium experiments.

Chloramphenicol and Tris. The effect of the antibiotic chloramphenicol and pH buffering by Tris on the anoxic resistance of *Scapharca inaequivalvis*, *Venus gallina*, *Tapes philippinarum* and *Mytilus galloprovincialis* was determined. At the start of the experiment, 48 (*V. gallina*) or 24 (all other species) specimens were added to anoxic medium in 1.5 l dark-brown glass bottles. The following media were used: (1) filtered anoxic seawater; (2) filtered anoxic seawater with the addition of 3 mmol l^{-1} Tris; (3) filtered anoxic seawater with the addition of 5 mg l^{-1} chloramphenicol; (4) filtered anoxic seawater with the addition of both 3 mmol l^{-1} Tris and 5 mg l^{-1} chloramphenicol. The pH was adjusted to ~8, the pH of the deoxygenated seawater. The bottles were held in the dark. The media were changed every 24 h and dead individuals were removed. Sulphide concentration and pH were regularly determined. The temperature was kept at 18°C.

Effect of chloramphenicol in the presence of Tris buffer and sulphide. In this experiment, the anoxic medium of both the control and the experimental groups contained initially about 0.4 mM H_2S and 20 mM Tris. The pH of the incubation medium was adjusted to 8.0 at the begin of the experiment. Chloramphenicol (5 mg l^{-1}) was also added to the experimental group at the beginning of the experiment and at 7 d intervals thereafter. The bivalves were placed in 4 l flasks at 18°C; the flasks contained 24 specimens of *Venus gallina*, or 8 specimens of *Scapharca inaequivalvis* or *Mytilus galloprovincialis* (in duplicate). Survival was checked daily, and the water was not renewed. H_2S concentration was frequently determined, and pH was checked after 1 wk and near the end of the experiment (Day 22). Two flasks without bivalves were filled with the medium of the control and experimental groups.

Determination of sulphide, ammonia and pH. Sulphide was determined after Svenson (1980) and ammonia after Truesdale (1971). The pH was measured using a Radiometer PHM 82 pH meter with a sulphide-insensitive electrode.

Statistics. The non-parametric Kaplan-Meier test was used to estimate log-rank and Wilcoxon values for comparing the survival curves (Kaplan & Meier 1958). A confidence limit of 95% was used to test the significance of differences between groups. LT_{50} values (median survival times) were estimated using the trimmed Spearman-Karber method ($\alpha = 10\%$) (Hamilton et al. 1977). In the case of replicates, results were pooled for the calculation of LT_{50} and for comparing survival curves.

RESULTS

Cadmium

Exposure to 100 µg Cd l^{-1} for 2 wk prior to incubation in Cd-free anoxic seawater caused a significant decrease in the anoxic survival time of *Scapharca inaequivalvis*. LT_{50} (11°C) of cadmium-exposed and control clams from the sandy sediment were 23.0 and 27.6 d, respectively (Fig. 1: upper graph). Surprisingly, acute exposure of *S. inaequivalvis* collected from the muddy sediment to 500 µg Cd l^{-1} in anoxic seawater affected the survival time positively. LT_{50} values (17°C) of the controls and cadmium-treated group were 16.1 and 27.3 d, respectively (Fig. 1: lower graph). After a few

days, the shells of the control group gradually changed colour from grey to black, while the cadmium-treated group retained their natural yellowish colour. However, *S. inaequivalvis* dredged from sandy sediment did not benefit from the presence of cadmium. There was no significant difference between the survival curves of the control and experimental groups. The LT_{50} (11°C) for these groups was 27.6 and 27.7, respectively (Fig 1: upper graph). Towards the end of the experiment, the shells of the controls were only slightly darker than that of Cd-treated groups. On the other hand, *Venus gallina* collected from sandy sediment benefited from the presence of cadmium in anoxic seawater, as demonstrated by an increase in the LT_{50} value (18°C) from 3.4 to 7.6 d (Fig 2). The control individuals developed a grey precipitate on their shell valves after the third day, while the Cd-treated individuals were seemingly unchanged.

Changes in incubation water

In this experiment, the median survival time of *Venus gallina* was 6.3 days (Fig. 3). There was a rapid increase of ammonia and sulphide in the incubation water, especially in the final stage, when mortality increased rapidly. At Day 6, there was 1.24 mmol l^{-1} ammonia and 0.31 mmol H_2S l^{-1} in the water (Fig. 3: upper graph). During the incubation period, the pH dropped by about 2 units (Fig. 3: lower curve in bottom graph).

Chloramphenicol and sulphide

Chloramphenicol was added to prevent biotic sulphide formation. For both *Scapharca inaequivalvis* and *Venus gallina*, survival time was prolonged by addition of chloramphenicol. In the previous experiments we obtained LT_{50} values (18°C) for *V. gallina* of 3.4
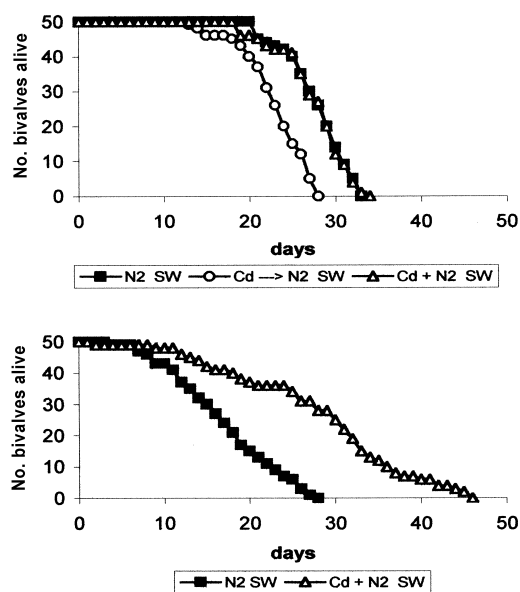

Fig. 1. *Scapharca inaequivalvis.* Survival in anoxic seawater of controls (N_2 plus seawater, N2 SW) and cadmium (500 ppb)-exposed (Cd+N2 SW) bivalves collected from sandy (upper graph) and muddy sediments (lower graph). 'Sandy' clams were also exposed to cadmium (100 ppb) for 2 wk prior to anoxic incubation (Cd→N2 SW: upper graph). The media were changed every 24 h; 'sandy' clams were incubated at 11°C, 'muddy' clams at 17°C
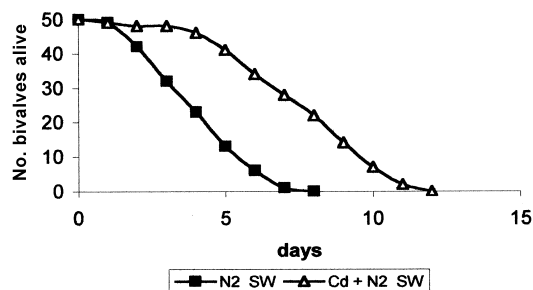

Fig. 2. *Venus gallina.* Survival in anoxic seawater of controls (N2 SW) and cadmium (500 ppb)-exposed (Cd+N2 SW) bivalves. The media were changed every 24 h. Temperature was 18°C
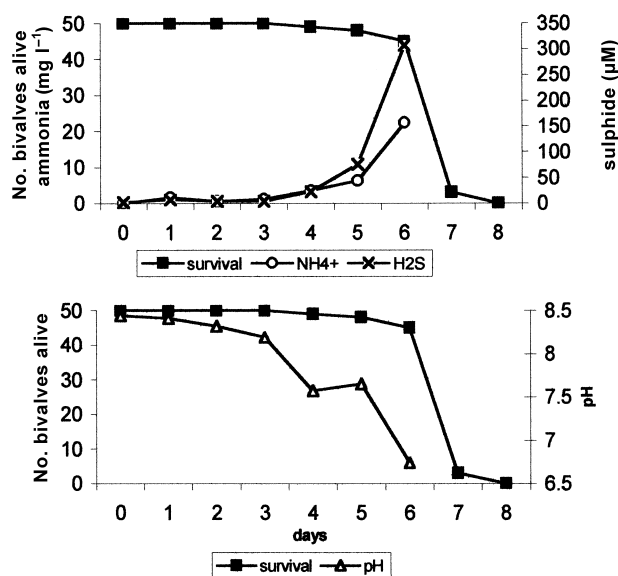
Fig. 3. *Venus gallina*. Anoxic survival (both graphs), accumulation of ammonia and sulphide (upper graph) and drop in pH (lower graph) in the anoxic seawater containing *V. gallina*. The incubation medium was not changed during the experiment; temperature was 18°C
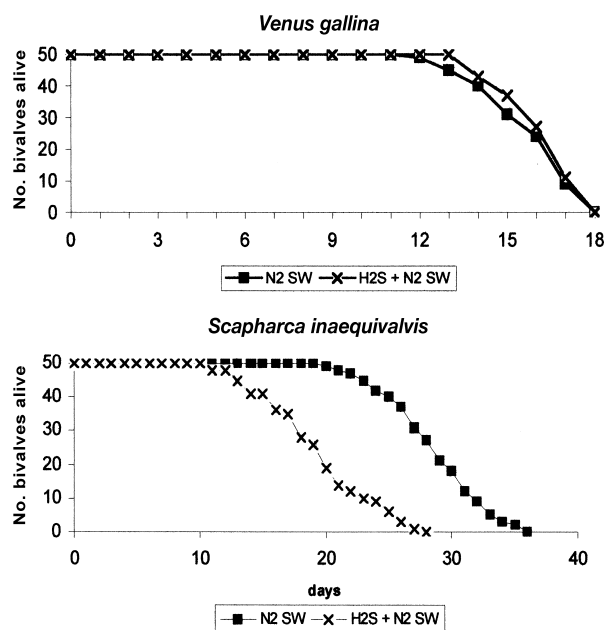

Fig. 4. *Venus gallina* and *Scapharca inaequivalvis*. Survival in anoxic seawater containing chloramphenicol of controls (N2 SW) and sulphide (H2S + N2SW)-exposed bivalves. The incubation media were changed every 24 h; temperature was 18°C

(Fig. 2) and 6.3 d (Fig. 3), compared with 16.2 d (chloramphenicol minus exogenous sulphide) in this experiment. Also, a strong effect of the antibiotic was observed with *S. inaequivalvis* (LT_{50} values from 16.1 d at 17°C to 28.2 at 18°C, Figs. 1 & 4, respectively).

The addition of 0.2 mmol H_2S l^{-1} to the anoxic seawater did not have a significant adverse effect on anoxic survival time for *Venus gallina*. Like the controls, 50% mortality was reached at about 16 d (Fig. 4). Simultaneous exposure of *Scapharca inaequivalvis* to anoxia and sulphide resulted in significantly lower LT_{50} values (18.6 d) in comparison to controls (28.2 d) (Fig. 4). The rapid blackening of shells of both species when sulphide was added was remarkable. In the blood clam, the gills were also noticeably blackened after half a day.

Chloramphenicol and Tris

In this comparative study, the 4 bivalve species showed widely different tolerances to lack of oxygen (Fig. 5). In anoxic seawater without additions (Medium 1), *Venus gallina* showed by far the lowest survival: anoxic survival time of the most tolerant species, the blood clam *Scapharca inaequivalvis*, expressed as LT_{50}, was about 4.5 times longer. The anoxic survival times of *Mytilus galloprovincialis* and *Tapes philippinarum* were intermediate. The addition of the antibi-

otic chloramphenicol (Media 3 and 4) led to a ~4-fold increase in survival time for *V. gallina* and a ~2- to 3-fold increase for the other species (Table 1, Fig. 5).

For all species, progressively increasing levels of sulphide could be detected in the incubation media without chloramphenicol with increasing incubation time, despite the fact that the medium was changed daily and dead individuals removed. This coincided with a gradual blackening of the shells. The accumulation of sulphide was strongest in *Scapharca inaequivalvis*, in which it exceeded the other species by at least 1 order of magnitude. The addition of chloramphenicol mar-

Table 1. *Scapharca inaequivalvis*, *Venus gallina*, *Tapes philippinarum* and *Mytilus galloprovincialis*. Effect of chloramphenicol (CA) in the presence or absence of Tris (3 mM) on LT_{50} values (d) of 4 bivalve species. Superscripts indicate comparison of corresponding survival curves; those with letters in common are significantly different at p < 0.05

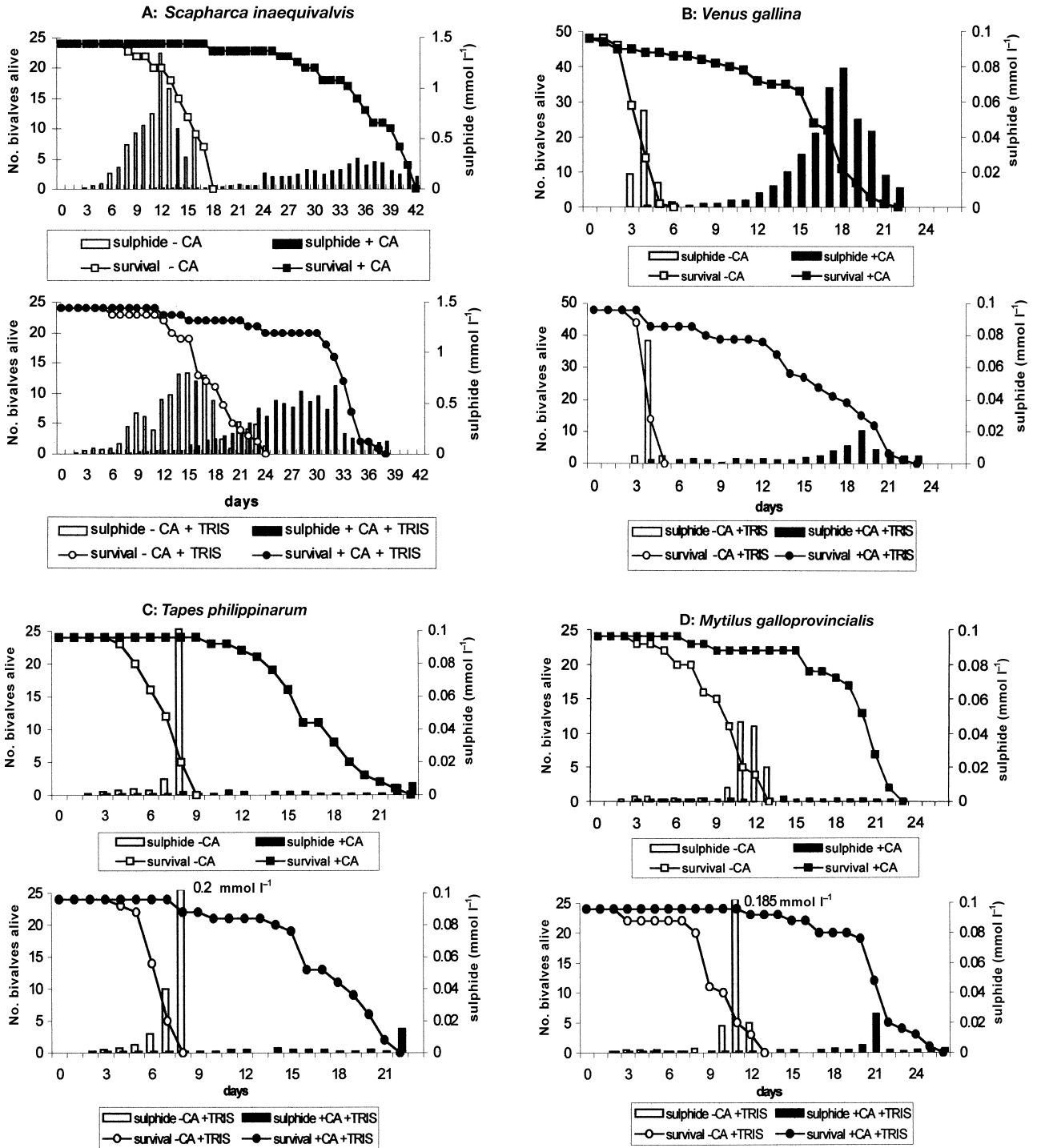| Species	Tris		No Tris	
	+CA	−CA	+CA	−CA
S. inaequivalvis	33.9[a]	17.0[b]	36.1[a]	14.7[b]
V. gallina	15.0	3.7	15.1	3.3
T. philippinarum	17.0	6.2	16.3	6.6
M. galloprovincialis	20.8	9.3	19.5	9.8

Fig. 5. *Scapharca inaequivalvis*, *Venus gallina*, *Tapes philippinarum* and *Mytilus galloprovincialis*. Survival of bivalves and accumulation of sulphide in non-buffered (upper graph) and Tris-buffered (+Tris; lower graph) anoxic seawater in the absence (−CA; open symbols) or presence (+CA; closed symbols) of chloramphenicol. The media were changed every 24 h; temperature was 18°C

kedly delayed sulphide formation and, with the exception of *Venus gallina* (without Tris), caused a decrease in the maximum concentrations reached (Fig. 5).

Except for the first 24 to 48 h, the anaerobic non-buffered incubations caused, for all species, a drop in the pH of the incubation medium of 1 to 2 units during
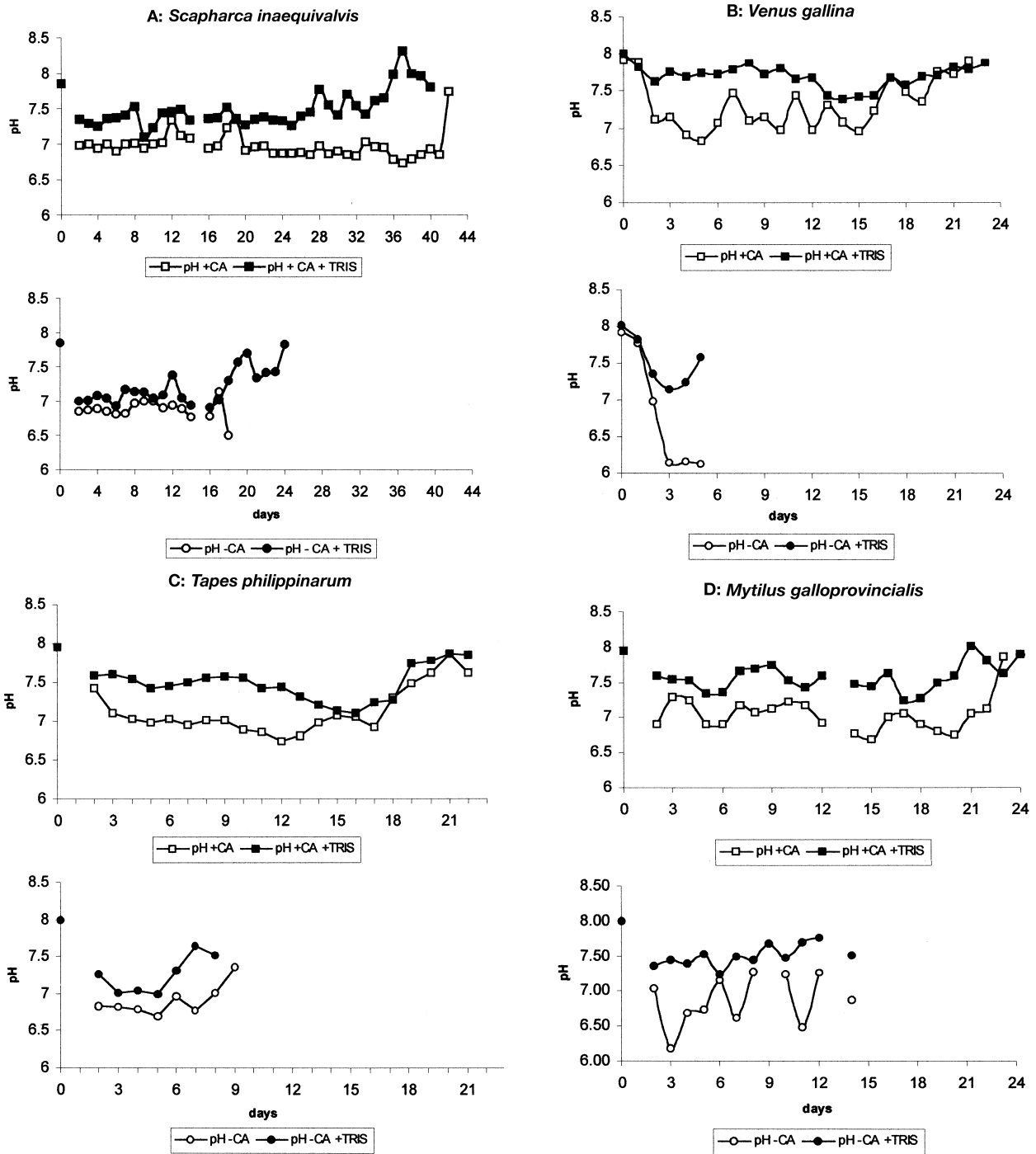
Fig. 6. *Scapharca inaequivalvis*, *Venus gallina*, *Tapes philippinarum* and *Mytilus galloprovincialis*. Changes in pH of non-buffered (open symbols) and Tris-buffered (+Tris; closed symbols) anoxic seawater in the presence (+CA; upper panel) or absence (−CA; lower panel)) of chloramphenicol, in survival experiments shown in Fig. 5

the 24 h between water renewal (Fig. 6), but only when the number of survivors was ≥80%. With fewer individuals, the drop in pH was smaller. For the 3 infaunal species, the addition of Tris buffer or the antibiotic decreased acidification, while both added simultaneously acted synergistically. However, under no condition was the drop in pH completely prevented. For *Mytilus galloprovincialis*, the addition of the buffer

reduced the drop in pH, but chloramphenicol had no effect on the acidification of the medium (Fig. 6D).

For *Venus gallina*, *Mytilus galloprovincialis* and *Tapes philippinarum*, buffering by Tris had no significant effect on survival time (Fig. 5B,C,D, Table 1). For *Scapharca inaequivalvis*, there was a small but significant difference. In the absence of chloramphenicol, Tris had a positive effect, in the presence of the antibiotic a negative effect (Fig. 5A, Table 1).

Chloramphenicol, Tris buffer and sulphide

In this experiment, the Tris buffer (20 mM) was able to maintain the pH near to the value to which the media were adjusted. Without chloramphenicol, the pH of the seawater control was 7.9 after 7 d; for the media containing *Scapharca inaequivalvis*, *Venus gallina* and *Mytilus galloprovincialis* it was 7.7, 7.6 and 7.9 respectively. When chloramphenicol was added, the pH in all cases lay between 7.8 and 7.9. At Day 22, the corresponding values were 7.9 for seawater and 7.0, 7.2 and 7.4 for the media with bivalves; when chloramphenicol was added, these values were 7.9 and 7.8, 7.6 and 7.9.

The initial concentration of ~0.4 mmol l^{-1} sulphide decreased after 24 d in the seawater controls to 0.29 (without chloramphenicol) and 0.31 mmol l^{-1} (with chloramphenicol). Also, in all media with bivalves, there was a decrease in the sulphide concentration which, however, never dropped below 0.22 mmol l^{-1}. When the bivalves were dying, there was an increase in sulphide concentration, especially in the absence of chloramphenicol (Fig. 7).

The results show clearly that the presence of sulphide in both the control and the experimental groups did not influence the relative LT$_{50}$ increase caused by the antibiotic (Fig. 7, Table 2). For the 3 species tested, the degree of LT$_{50}$ increase corresponded well with that in the previous experiment (Table 1). The absolute values for LT$_{50}$ in this experiment were slightly higher for *Mytilus galloprovincialis* and *Venus gallina*. This was possibly the result of a larger medium-to-animal ratio and less handling of the bivalves (no daily

Table 2. *Scapharca inaequivalvis*, *Venus gallina* and *Mytilus galloprovincialis.* Effect of chloramphenicol (CA) in the presence of Tris (20 mM) and H$_2$S (0.4 mM) on LT$_{50}$ values (d) of 3 bivalve species

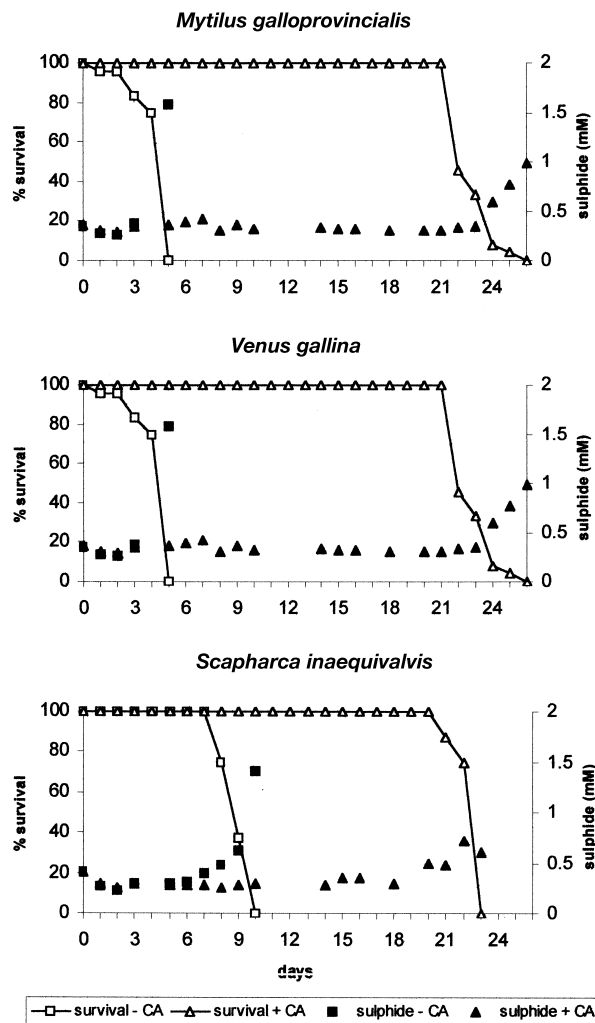| Species	+CA	−CA
S. inaequivalvis	22.2	8.6
V. gallina	22.3	4.1
M. galloprovincialis	21.7	11.9

Fig. 7. *Mytilus galloprovincialis*, *Venus gallina* and *Scapharca inaequivalvis.* Survival (□, △) and sulphide concentrations (■, ▲) in anoxic, Tris-buffered seawater containing sulphide (initial 0.4 mM) of controls (−CA; □, ■) and chloramphenicol-exposed (+CA; △, ▲). The incubation medium was not changed during the experiment

medium renewal). This is in sharp contrast with the result for *Scapharca inaequivalvis*, for which the median survival time both in the presence and absence of chloramphenicol appeared to be strongly negatively affected by the added sulphide. This agrees with the previous result on the effect of sulphide on survival time of *S. inaequivalvis* and *V. gallina*, and indicates that, like *V. gallina*, *M. galloprovincialis* is insensitive to the presence of sulphide in the medium.

DISCUSSION

Laboratory and field studies have demonstrated the applicability of anoxic survival as an early warning

indicator of contaminant-induced stress. Following chronic exposure to sublethal toxicant concentrations, anoxic survival is generally decreased (de Zwaan & Eertman 1996). Also, exposure of *Scapharca inaequivalvis* to 100 ppb Cd for 2 wk prior to transfer to anoxic seawater resulted in a decrease in anoxic survival time. However, when *S. inaequivalvis* (collected from muddy sediment) and *Venus gallina* were exposed to a 5 times higher dose of Cd under anoxic conditions, survival time increased by a factor of 2 (Figs. 1 & 2). Blackening of the bivalve shells was observed in anoxic water in the absence of Cd, but not when Cd was present. The blackening of the shells in the controls points to the generation of sulphide by microbial activity, which seemed to be inhibited by Cd. In the case of the controls, the microbial release of sulphide (and other noxious products) may have caused adverse effects on LT_{50}. For *S. inaequivalvis* collected from sandy sediment, shell blackening was rarely observed, pointing to less contamination by sulphide-producing bacteria. In addition, the lower incubation temperature of 11°C (opposed to 17°C) for the 'muddy' incubated clams probably slowed down bacterial proliferation.

The blackening of shells was reduced in all experiments in which bacterial growth was reduced, either by natural factors correlated with the location of growth (Fig. 1: upper graph) or by addition of antibiotics. The possibility that sulphide produced by bacteria might have abiotically complexed with Cd can be ruled out. First, the added concentration of Cd (500 µg l^{-1} = ~4 µmol l^{-1}) is at least 1 order of magnitude lower than the spontaneously formed concentrations of sulphide (see e.g. Figs. 3 & 5). Second, a comparable positive effect on survival time was obtained when *Scapharca inaequivalvis* was incubated in the presence of 500 ppb Cd for 2 wk prior to the anoxic survival test in Cd-free anoxic seawater (results not shown).

Shell blackening is caused by precipitation of a metal and sulphide, and absence of discolouration points to inhibition of sulphide formation. This was an indication that the adverse effect on anoxia tolerance is caused by the growth of anaerobic bacteria. Further studies, in which a broad-band antibiotic (chloramphenicol) was used to delay bacterial growth confirmed strong correlations between the occurrence of bacteria, monitored as sulphide production, and increased mortality of bivalves. In a pilot study, we also obtained positive effects on survival with oxytetracycline hydrochloride, streptomycin sulphate and penicillin-G (separate and in combination). All 4 bivalve species showed strongly improved anoxic survival times in its presence (2- to 4-fold: Table 1). Especially for *Venus gallina*, but also for *Scapharca inaequivalvis*, the benefit was higher than with Cd. Without antibiotic, sulphide accumulation was observed for all spe-

cies (Fig. 5). In the case of the blood clam, the presence of dead individuals was no prerequisite for the formation of sulphide. From Day 2 up to Day 13, there was an exponential increase in sulphide concentration (up to 1.35 mmol l^{-1}), whereas the first clam died on Day 8 (Fig. 5A: upper curve). Accumulation not only began much earlier than for the other species, but also reached much higher concentrations. The antibiotic was able to postpone the accumulation of sulphide considerably, but did not finally prevent it. Seemingly protected by the presence of the bivalve, a significant population of sulphate-reducing bacteria was able to survive in the presence of chloramphenicol. As in other bivalves, this apparently chloramphenicol-resistant population increased when the bivalves began to die, and was removed from the flask together with the dead individuals. However, we cannot conclude from these data that sulphide caused the death of the bivalves, because mortality in the presence of sulphide (Figs. 4 & 7) did not increase at the onset of sulphide treatment, but only when endogenous sulphide-production indicated excessive growth of sulphate-reducing bacteria. Although we cannot exclude the possibility that a small number of chloramphenicol-resistant sulphide-producing bacteria were associated with the bivalves initially, it is more likely that these bacteria proliferate in places where access of the antibiotic is limited, e.g. possibly in biofilms or in (necrotic) tissue of the bivalve. Decomposition of the antibiotic can be ruled out, as it was added freshly each day during renewal of the medium.

The responsible micro-organisms must have been introduced with the bivalves and not with the seawater. This follows from the fact that progressively increasing levels of sulphide could be detected in the incubation medium (Fig. 5), despite the fact that the medium was exchanged daily. It also explains why the levels reached a maximum and decreased again when the number of bivalves decreased through mortality. Anoxia obviously induced the proliferation of bacteria, which remained associated with the bivalves. Regular renewal of the incubation medium in order to avoid accumulation of toxic metabolites (see e.g. Oeschger & Pedersen 1994, Jahn & Theede 1997) may therefore not be effective.

Both the bivalves and micro-organisms must have been responsible for acidification of the medium (Figs. 4 & 6). Acidification was strongest in the absence of the antibiotic and the buffer. Acidification in the presence of the antibiotic must have been mainly due to the excretion of acids produced by anaerobic catabolism of the bivalves. Possible candidates are CO_2, acetate and propionate (de Zwaan et al. 1991). The pH values for all incubations ranged between 6 and 8. A lower pH value had no or inconsistent effects on sur-

vival time. The positive result of chloramphenicol on anoxic survival was therefore independent of its effect on acidification.

In the final experiment (Fig. 7) the main objective was to investigate the extent to which the positive effect of chloramphenicol on anoxic survival could be counteracted by sulphide. Therefore, a relative high dose of sulphide (400 µM) was added to both the control and the experimental groups. Chloramphenicol was added only to the experimental group. As expected, *Venus gallina* again showed a much longer anoxic survival time as a result of chloramphenicol addition, despite the addition of sulphide. We have already shown that this organism was insensitive to a high dose of sulphide (Fig. 4). Also in the case of *Scapharca inaequivalvis* and *Mytilus galloprovincialis*, the relative increase in survival time due to chloramphenicol remained in combination with a high dose of sulphide (Fig. 7). Only for the blood clam was the absolute survival time of both the control and the experimental groups strongly reduced. For this species, suppression of sulphide generation can therefore explain part of the positive action of the antibiotic (and the high dose of Cd). This means that additional (blood clam) compounds or compounds other than sulphide must have been responsible for the adverse effects on survival of the controls, and/or that bacteria acted directly in disrupting the organism.

It is intriguing that *Scapharca inaequivalvis* showed the highest sulphide production indicating association with the largest population of sulphate-reducing bacteria. Furthermore, this bivalve apparently provided protection to sulphate-reducing bacteria against chloramphenicol before it died (Fig. 5). It is tempting to speculate that this bivalve has a benefit from these bacteria, but the mechanism is not obvious. In our assays, *S. inaequivalvis* was the only bivalve that exhibited a significant decrease in survival time in the presence of sulphide (Figs. 4 & 7), a major product of sulphate-reducing bacteria. Furthermore, the latter are obligatory anaerobic, heterotrophic bacteria, while all bacterial symbionts described for diverse marine invertebrates are chemoautotrophic sulphide (or methane)-oxidising bacteria that simultaneously provide, food and sulphide detoxification (Distel 1998). It has been assumed that a fairly high concentration of sulphide is a prerequisite for bivalve association with symbiotic sulphur bacteria (i.e. deep-sea hydrothermic vents, sewage outfalls and seagrass beds). Dando et al. (1985) showed the presence of sulphur-oxidising bacteria in the gill of the bivalve *Myrtea spinifera* living in muddy sand of a Norwegian fjord, but the sediment in which this bivalve lives has a very low concentration of free sulphide. This species possesses haemoglobin in its gill tissue, which has a high affinity for oxygen but lacks red blood cells. The blood clam *S. inaegiuvalvis* belongs to the Arcidae family which, unlike other molluscs, contains haemoglobin-laden erythrocytes (Chiancone et al. 1981). Also, its gills are red-coloured due to the presence of intracellular haemoglobin. Preliminary studies (by A. Hernandez at the Netherlands Institute of Ecology) of the bacterial populations associated with *S. inaegiuvalvis* have shown that sulphide-oxidising and sulphate-reducing bacterial populations occur not only in externally association with apparently healthy individuals of this bivalve, but also appear to cohabit in different tissues, even in the blood system. Possibly clams from low-sulphide sediments need to host sulphate-reducing bacteria to provide sulphide in order to profit from symbiosis with sulphide-oxidising bacteria. The beneficial effect of chloramphenicol under anoxic conditions (Figs. 4, 5 & 7) may indicate that opportunistic bacteria, which become pathogenic under severe stress, exist among the associated bacteria. Further studies on the quantification and identification of dominant species among associated bacteria is part of our current research program.

In conclusion, it is obvious that especially during prolonged anoxia and at high temperatures, the study of anoxic resistance in a closed system or even in a system with daily water changes may result in strongly underestimated survival times due to bacterial proliferation. Anoxic survival is therefore no reliable biological-response parameter for assessing water quality when the contaminants present display bacteriostatic effects. The biotic formation of sulphide plays a minor (blood clam) or no role in their adverse effect on survival. Studies on stoichiometric aspects of metabolism, including release of end-products, may also lead to artefacts. In such studies the addition of antibiotics should be considered. They are often applied in research on the anaerobic metabolism of worms (see e.g. Zebe 1975, Surholt 1977, Bestwick et al. 1989) and midge larvae (Englisch et al. 1982), but in general not in research on other aquatic invertebrates. There is a risk that the antibiotic itself interferes with metabolism. We sometimes observed for S*capharca inaequivalvis* the production of mucus in response to the presence of chloramphenicol in the water. Despite the remarkable positive effect of this antibiotic on survival, we cannot therefore exclude the possibility that the established high LT_{50} values for anoxia still underestimate the capacity of bivalves to survive anoxia.

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