Marine Biology (2001) 138: 1021–1028

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Studies on the causes of mortality of the estuarine bivalve *Macoma balthica* under conditions of (near) anoxia

Received: 4 July 2000 / Accepted: 6 December 2000

Abstract Survival of the bivalve *Macoma balthica* in (near) anoxic seawater was studied in a static system and a flow-through system and compared with emersed exposure to air and N_2. In the static system, a decrease in pH and exponential accumulation of sulphide in the incubation medium were observed, indicating excessive growth of (sulphate-reducing) bacteria. These changes in the chemical environment were prevented by the use of a flow-through system. However, this treatment hardly affected survival time. Median mortality times were 8.3 and 9.0 days for the static and flow-through incubation, respectively. Addition of the antibiotic chloramphenicol strongly increased survival time in both systems with corresponding values of 17.9 and 23.0 days. A similar value was obtained for survival in air (LT_{50} = 21.7 days). In a second experiment (1 year later), we obtained much lower values for anoxic survival in a static system, although laboratory conditions, season and temperature were similar. The pH values were adjusted to 6.5, 7.2 and 8.2 by buffering the media (25 mM Tris-HCl), and the corresponding LT_{50} values were 5.5, 5.7 and 4.7 days, respectively. In the presence of chloramphenicol the values were 10.8, 10.9 and 9.5 days, respectively. These values show that a slightly acidic medium increased survival time. Exposure to an atmosphere of N_2 resulted in a survival time close to that in anoxic seawater without chloramphenicol (LT_{50} = 6.4 days). Overall the results indicate that proliferation of anaerobic bacteria associated with the bivalves was the main cause of death. Since chloramphenicol also displayed a strong positive effect in the flow-through system, which prevented the accumulation of released waste products and a decrease of pH, bacterial damage must have been by injury of the tissues of the clams and not by the release of noxious compounds to the medium. Bacterial outbreaks are a part of every anoxic event (eutrophication), and therefore, in their habitats, direct bacterial infection may also be the cause of clam mortality. It is concluded that laboratory studies on anoxic tolerance, or impact of sulphide, may produce artefacts when no precautions are taken to suppress bacterial proliferation.

Communicated by O. Kinne, Oldendorf/Luhe

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Introduction

Knowledge of the tolerable duration of exposure to anoxia is important for modelling the impact of poor water quality. Without accurate estimates of how long anoxia must persist to kill benthic prey organisms, the true impact of eutrophication on fishery habitats cannot be modelled adequately and incorporated in water quality management decisions.

A large number of studies deal with anoxia tolerance of aquatic invertebrates (Theede et al. 1969; Rosenberg et al. 1991; Diaz and Rosenberg 1995; de Zwaan and Eertman 1996; Modig and Ólafsson 1998). These organisms show very distinct capabilities to survive anoxia even within taxonomically closely related groups. However, tolerance values of a single species also showed large variance between studies, probably due to variations in experimental conditions. Most studies were carried out in closed incubation devices without water renewal (static system) (Theede et al. 1969; Dries and Theede 1974), some with regular water renewal (semi-static system) (Jahn and Theede 1997) and a few in flow-through systems (Putzer et al. 1990; Levitt and Arp 1991). Understanding of the actual mechanisms causing death may help to explain the inconsistency in such literature data.

In a closed system without water renewal, proliferation of anaerobic bacteria in the incubation medium

may occur with adverse effects on anoxic survival (Felder 1979; Groenendaal 1980; Llansó and Diaz 1994). The general opinion is that bacterial interference is caused by the release of noxious compounds. In that case, the use of a flow-through incubation device should counteract the negative effects caused by accumulation of these compounds. In fact, in such a set-up Putzer et al. (1990) obtained a good balance between glycogen stored, anaerobic utilisation rate and survival time for the oligochaete *Lumbriculus variegatus*. A complication with larger macrofaunal species is the high amount of anoxic medium needed in a flow-through system, and this probably explains the general preference for static systems. However, when studying four bivalve species from the Mediterranean zone in closed systems, we observed that the experimental set-up had a great impact on survival time (de Zwaan et al. 2001a).

In order to gain more insight into how bacteria cause negative effects, we compared in the present study survival time in a static system with that in a flow-through system in the presence and absence of chloramphenicol. The flow was adjusted to a rate sufficient to remove toxins and metabolites by recording pH and sulphide concentrations, parameters established as indicators of bacterial growth in the static system. In a recent study we made estimations of the number of bacteria growing in anoxic semi-closed systems containing *Macoma balthica*. High numbers were counted in the controls and very low numbers in the presence of chloramphenicol. In the latter case there was virtually no biotic formation of sulphide, while pH decrease was diminished (de Zwaan et al. 2001b). To discriminate between a direct effect of pH on the bivalve and on bacterial growth, anoxic survival time was determined in static systems filled with seawater containing a buffer (Tris-HCl) adjusted to different pH values in the presence and absence of chloramphenicol. Anoxic survival in seawater was further compared with exposure to air and an atmosphere of N_2.

For practical reasons, we selected a relatively small bivalve species for this study, the Baltic clam *M. balthica*, which is generally present in the mudflats of Dutch coastal waters (Temperate Zone). This bivalve is often a biomass dominant in estuaries on both sides of the Atlantic and is very important trophically as prey for demersal fishes and some waterfowl (Bachelet 1980; Beukema and Meehan 1985).

Materials and methods

Individuals

Infaunal specimens of *Macoma balthica* were sampled from muddy sediments between mean tidal level and low water level at Paulinapolder, a tidal flat of the western Scheldt estuary in the southwestern part of The Netherlands. Clams were dug up and sifted out. Average shell lengths of the clams were 16.1 ± 1.9 mm (average and standard deviation; $n = 50$). The average total wet weight was 0.41 g, and the shell volume 0.35 ml. Salinity at the sampling site was 27 ± 2 psu, and temperature between 17 °C and 19 °C. The clams were kept in an aquarium with sterilised sandy sediment and well-aerated unfiltered running seawater of 17 °C and 31 psu, pumped in from an inlet in the eastern Scheldt in front of the institute at Yerseke. On day 4 filtered seawater was used to purge the animals. The next day the clams were used in the experiments. Mortality was assessed by failure of constriction when touching the mantle edge of gaping animals with an iron rod. Dead animals were removed from the incubation containers. No food was added during anoxic incubations.

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

Specific determinations and statistics

Sulphide was determined after Svenson (1980). The pH was measured using a Radiometer PHM 82 pH meter with a sulphide-insensitive electrode.

The non-parametric Kaplan–Meier test was used to estimate log-rank and Wilcoxon values for comparing the survival curves (Kaplan and 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).

Protocol of experiments

Flow-through versus static incubation and exposure to air

Figure 1 gives a schematic drawing of the flow-through set-up. Filtered anoxic incubation medium was pumped from a 60 l reservoir through the animal container (13 cm inner diameter, 26 cm height, ~1200 ml volume) with a flow of 9 ml min^{-1}, resulting in exchanges of ~10 vol day^{-1}. Three reservoirs contained different media: (a) filtered anoxic seawater of pH 8.2; (b) filtered anoxic seawater with the addition of 25 mmol Tris l^{-1}, adjusted with HCl to pH 8.2; (c) filtered anoxic seawater with the addition of 3 mg chloramphenicol l^{-1}, adjusted to pH 8.2. The media were constantly bubbled with N_2, and a tube outlet with small diameter maintained excess pressure in order to ensure that no air could enter the system. Every third day the reservoirs were refilled, while the pump was stopped for 2 h to allow removal of oxygen by vigorously bubbling with N_2. At a small distance from the top of the incubation containers, a rack was mounted to support 50 animals. Two holes in the top of the containers, which normally were closed by rubber stops, served to remove dead animals and to measure concentration of oxygen (Beckman oxygen analyser). *Oxygen concentration never exceeded 0.20 mg* l^{-1}.

For static incubations, identical incubation containers were filled at the same time with medium a or medium c, and subsequently the inlet and outlet holes were blocked with rubber stoppers. In these containers, the media were not exchanged, but, to the container filled with medium c, chloramphenicol (6 mg l^{-1}) was added as dry powder every fifth day. In a semi-static anoxic incubation in which the medium was exchanged every 3 days similar effects on survival were obtained with 3 and 6 mg chloramphenicol l^{-1} (pilot-experiment). The first dose was used in the flow-through systems and the latter (repetitively) in the static systems in order to compensate for possible decomposition of the compound. Oxygen concentrations never exceeded 0.15 mg l^{-1} (Winkler method). At the start of the experiment, and daily once animals started to die, pH and sulphide concentration of the medium were measured.

To estimate tolerance at aerial exposure in the same experiment, three incubation containers were partly filled with seawater until the surface was just below the rack with clams. The holes in the top were not closed, and the air saturated by water vapour was flushed through the upper part of the vessel (aerial exposure). The experiment was started by placing 50 animals in one layer on the rack.

All above incubations were carried out as one experiment, conducted in autumn 1998 at 17 °C.

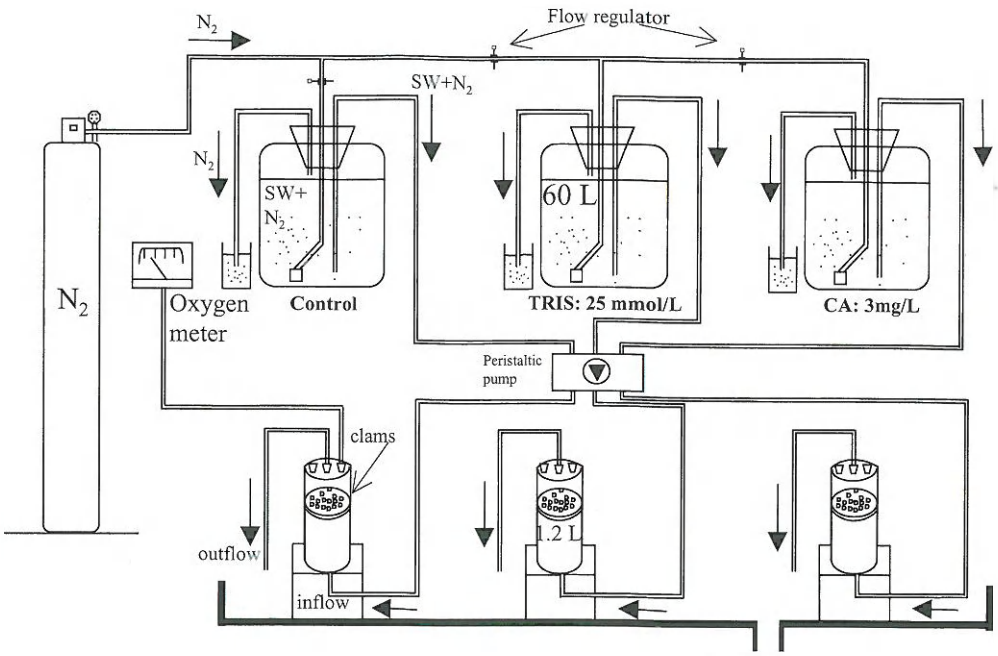

Fig. 1 Diagram showing the flow-through set-up. The incubation media in the storage containers were constantly bubbled with N_2 and passed by a peristaltic pump through the incubation chambers holding 50 clams each. Medium consumption was compensated by a refill every 3 days. For details see "Materials and methods"

Effect of pH on anoxic survival in a static system and exposure to nitrogen

Two sets of three conical flasks were filled with 1 l anoxic seawater containing 25 mM Tris adjusted with HCl to pH 6.5, 7.2 and 8.2. In one set also, 6 mg chloramphenicol l^{-1} was added to each flask. At the start, 50 specimens were introduced to each flask, which covered part of the bottom. Mortality and pH were checked daily, and sulphide regularly.

Anoxic survival in a water-saturated nitrogen atmosphere was determined by placing 50 clams on a rack just above the water in an incubation container (as described above), a constant stream of N_2 being bubbled through the water. Mortality was checked daily. This experiment was carried out in autumn 1999 at 17 °C.

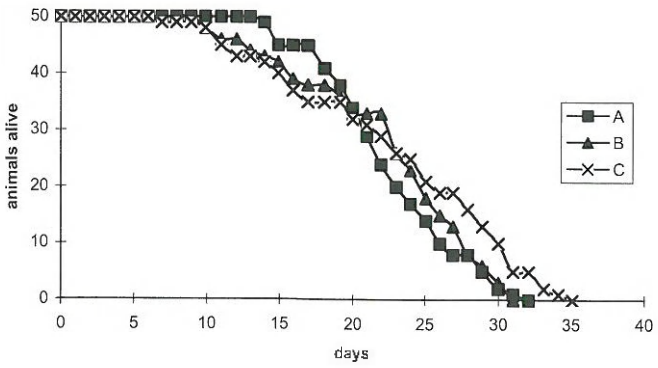

Fig. 2 *Macoma balthica*. Survival in water-saturated air. Profiles are shown of three replicates (*A–C*) with 50 clams each

Results

Figure 2 shows the survival of three replicates of 50 clams exposed to air. Mean (± SD) median mortality value (LT_{50}) was 21.65 ± 0.21 days. No significant differences were found between the replicates ($P > 0.05$ for all comparisons), showing that a group size of 50 clams gave reproducible results.

In Fig. 3 the survival of clams in static incubations was compared to that in flow-through incubations and during aerial exposure. A possible effect of Tris at 25 mmol l^{-1} was examined in the flow-through system. The curves can be roughly divided into two groups of low and high survival time. Low survival times were observed in the static system and flow-through system (with and without Tris). High survival time was observed in anoxic seawater containing chloramphenicol (static and flow-through system) and during exposure to air. The first group has an average LT_{50} value of 8.6 days, the values being within a narrow range of 8.3–9.0 days. The second group shows a 2.4-fold

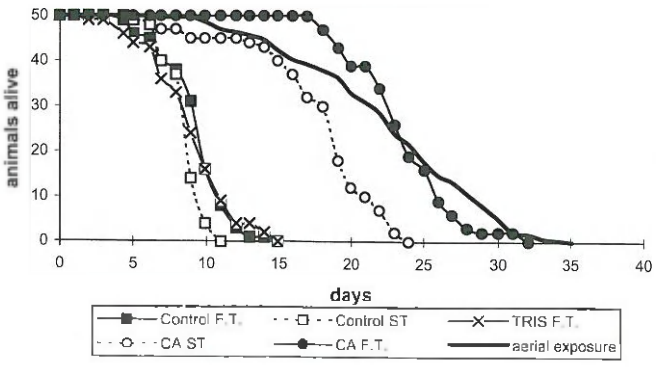

Fig. 3 *Macoma balthica*. Survival of clams exposed to air (average of the three replicates in Fig. 2) and anoxic seawater in static systems (*ST*) and flow-through systems (*F.T.*). The aquatic incubations were carried out in the absence and presence of chloramphenicol (*CA*). One flow-through incubation was carried out with 25 mM Tris (*TRIS*). In all cases pH was adjusted to 8.2

increase in survival time (average LT_{50} value = 20.9 days), values ranging from 17.9 to 23.0 days (Table 1). Surprisingly, life prolongation by exposure to air was similar to the effect of the antibiotic to anoxic water in the flow-through system ($P > 0.05$; Table 2).

In the static control, sulphide accumulated to 400 µmol l^{-1} during the period of mortality. This was blocked (sulphide concentration <3 µmol l^{-1}) by the presence of chloramphenicol (Fig. 4A). Also the pH dropped in 12 days from 8.2 to 6.7 in the static incubation. Total pH decrease in the presence of chloramphenicol was diminished and delayed (from 8.2 to 7.0 in 24 days; Fig. 4B).

In the flow-through incubations sulphide accumulation remained virtually absent (<4 µmol l^{-1}; Fig. 5A), and the pH close to 8.2 (Fig. 5B). This means that the flow rate was sufficient to flush away (noxious) products released by the clams and microorganisms. As for the static incubations, the presence of chloramphenicol substantially increased survival time (Fig. 5; Table 1).

The flow-through medium containing the Tris-HCl buffer was adjusted to pH 8.2, the same pH as measured for natural anoxic seawater (controls). Due to the continuous flow of both media, the pH remained at this level throughout the experiment (Fig. 5B). LT_{50} values were 9.0 and 8.5 days for the control and the medium containing Tris (Table 1), respectively, and the survival curves were not significantly different ($P > 0.05$; Table 2).

As Tris in the concentration used (25 mmol l^{-1}) had no apparent toxic effect in the flow-through system,

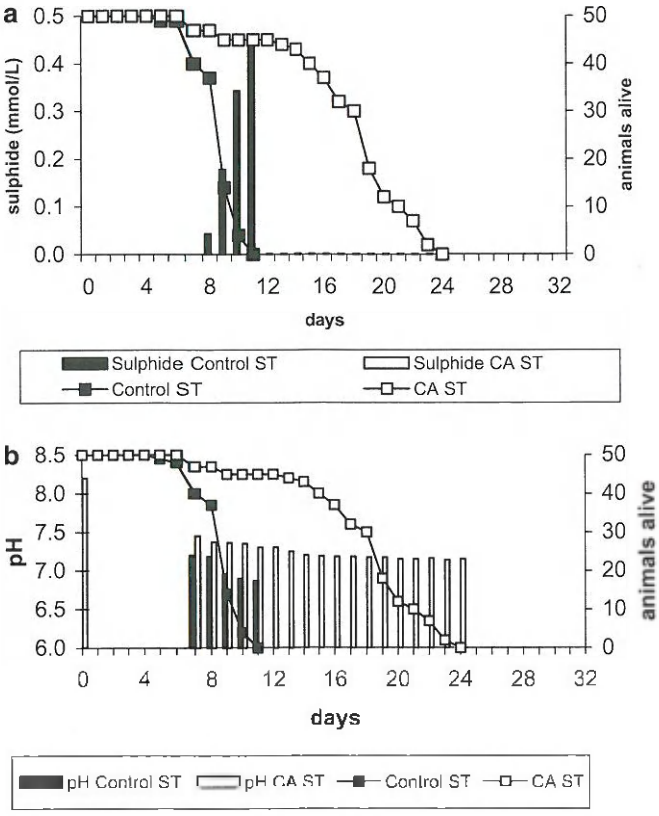

Fig. 4A, B *Macoma balthica*. Survival of clams and changes in the concentration of sulphide (**A**) and pH (**B**) in static incubations (*ST*) without (*Control*; *closed symbols*) and with chloramphenicol (*CA*; *open symbols*). Survival is shown by *curves*, sulphide and pH values by *bars*. (The bars for pH at day 0 represents both control and CA)

Table 1 *Macoma balthica*. Median survival times (LT_{50} values) calculated with the trimmed Spearman–Kraber method for aerial emersion and for incubation under aquatic anoxia in static and flow-through systems in the absence and presence of chloramphenicol (CA) (see also Fig. 3)

	Flow-through			Static		Air
	Control	Tris	CA	Control	CA	
Days	9.0	8.5	23.0	8.3	17.9	21.7

Table 2 *Macoma balthica*. Comparison of the survival curves in Fig. 3 (corresponding abbreviations) with the non-parametric Kaplan–Meier test (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; *NS* not significant at $P > 0.05$)

	Flow-through (F.T.)			Static (ST)		Emersed
	Control	Tris	CA	Control	CA	Air
Control F.T.						
Tris F.T.	NS					
CA F.T.	***	***				
Control ST	**	NS	***			
CA ST	***	***	***	***		
Aerial exposure	***	***	NS	***	***	

1 year later we examined survival of clams in a static system at different pH levels, by incubating in media adjusted with Tris-HCl buffer to pH 6.5, 7.2 and 8.2. Survival times were much lower as compared to the static incubations in the initial experiment, although temperature and season were the same.

In Fig. 6A the survival curves at the three pH values are shown. The median mortality time was 5.5, 5.7 and 4.7 days, respectively (Table 3). Survival time at pH 8.2 was significantly lower than at both lower pH values ($P < 0.001$, Table 4). Once mortality of the clams started, an increase in sulphide levels was observed, indicating growth of sulphate-reducing bacteria (Fig. 6B). This was most pronounced at pH 8.2. When chloramphenicol was added, survival at pH 8.2 was also significantly lower than at lower pH values ($P < 0.05$, Table 4), although the difference was smaller. In this case bacterial growth, as judged from sulphide concentrations, was virtually absent (Fig. 6B). The corresponding LT_{50} values were 10.8, 10.9 and 9.5 days, respectively (Table 3).

Exposure to nitrogen atmosphere resulted in a survival time ($LT_{50} = 6.4$ days) closer to those in anoxic seawater than in anoxic seawater with chloramphenicol (Table 3; Fig. 6A).

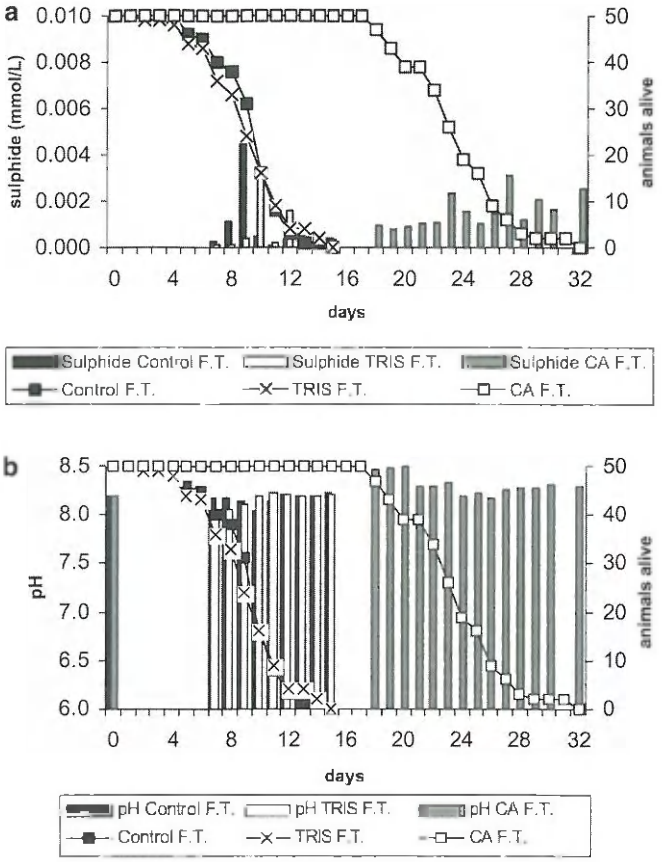

Fig. 5A, B *Macoma balthica*. Survival of clams and changes in the concentration of sulphide (**A**) and pH (**B**) in flow-through incubations (*F.T.*) without (*Control*; *closed symbols*) and with chloramphenicol (*CA*; *open symbols*). *TRIS* stands for control with addition of 25 mM Tris, pH 8.2. Survival is shown by *curves*, sulphide and pH values by *bars*

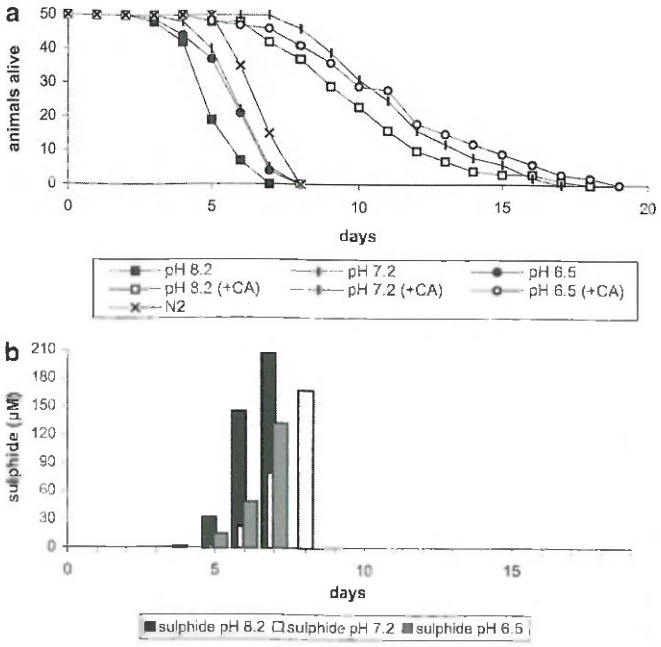

Fig. 6A, B *Macoma balthica*. Survival of clams (**A**) and sulphide accumulation (**B**) in anoxic seawater with 25 mM Tris adjusted with HCl to pH 6.5, 7.2 and 8.2. Survival rates in the absence (*closed symbols*) and presence of chloramphenicol (*CA*; *open symbols*) as well as in an atmosphere of nitrogen (*crosses*) are given. Sulphide accumulation up to the eighth day of incubation in the absence of choramphenicol is shown by *bars*. In the presence of chloramphenicol sulphide concentrations never exceeded 3 µM (not visible)

Discussion

Past reports on the mortality rate of *Macoma balthica* show wide variability in anoxic survival. Brafield (1963) observed that this clam only tolerated anoxia for 2 or 3 days (temperature not given). Dries and Theede (1974) reported LT_{50} values of specimens collected in the western Baltic Sea of 22 and 20 days at 10 °C and 15 °C, respectively. More recently, Jahn and Theede (1997) reported median mortality values of 8 to 12 days at 10 °C for clams from the same location.

Different studies on survival of *M. balthica* in drained sediment cores taken from Balgzand, an intertidal flat area in the north-western part of The Netherlands, showed strong differences in survival times, ranging from 7 to 55 days. Ambient air temperatures and glycogen contents were concluded to be important factors in determining the mortality rate (Hummel et al. 1986a , b; Fortuin et al. 1989). Finally, in the current study, we have established survival times for *M. balthica* of 8.3–9 days in 1998 (Table 1, autumn 1998) and 4.7–5.7 days in 1999 (Table 3, autumn 1999) at 17 °C. In both years clams came from the same collection site, and season and temperature were similar. This illustrates that factors related to a (long) history of events in the field must have had an impact on the outcome of our studies.

The large variation in data for *M. balthica* raises the question whether the results obtained in closed systems indeed reflect tolerance to anoxia, or artefacts. During incubation in the current study the shells gradually coloured from grey to black, and towards the end of the experiment even the incubation seawater turned grey-black. Also in the field the number of bivalves with grey and black colour increases when oxygen becomes depleted (Oeschger and Theede 1988). This pointed to microbial formation of sulphide which was confirmed by measurements (Fig. 4A). The pH of the medium decreased by about 1.5 units (Fig. 4B). Other changes in anoxic incubation media have been reported, such as

Table 3 *Macoma balthica*. Median survival times (LT_{50} values) calculated with the trimmed Spearman–Kraber method for emersion in nitrogen atmosphere and anoxic seawater buffered with 25 mM Tris-HCl at pH 6.5, 7.2 and 8.2, in the absence and presence of chloramphenicol (see also Fig. 6A)

	Control			Chloramphenicol			N_2
	pH 6.5	pH 7.2	pH 8.2	pH 6.5	pH 7.2	pH 8.2	
Days	5.5	5.7	4.7	10.8	10.9	9.5	6.4

Table 4 *Macoma balthica*. Comparison of the survival curves in Fig. 6 (corresponding abbreviations) with the non-parametric Kaplan–Meier test (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; *NS* not significant at $P > 0.05$)

	Control			Chloramphenicol			N_2
	PH 6.5	pH 7.2	pH 8.2	pH 6.5	pH 7.2	pH 8.2	
pH 6.5							
pH 7.2	NS						
pH 8.2	**	***					
pH 6.5 (+CA)	***	***	***				
pH 7.2 (+CA)	***	***	***	NS			
pH 8.2 (+CA)	***	***	***	*	*		
N_2	***	***	***	***	***	***	

increased concentrations of CO_2 and ammonia (Llansó and Diaz 1994). It is generally assumed that the accumulation of metabolic wastes will have an adverse effect on anoxic survival time (Felder 1979; Groenendaal 1980; Llansó and Diaz 1994). For sulphide the data for bivalves are contradictory. For *Venus gallina* (de Zwaan et al. 2001a) and *M. balthica* (Jahn and Theede 1997) the addition of sulphide did not reduce anoxic survival time, in contrast to *Scapharca inaequivalvis* (de Zwaan et al. 1993, 2001a), *Macoma secta* and *M. nasuta* (Levitt and Arp 1991) and *Mulinia lateralis* (Shumway et al. 1983).

In the flow-through system the continuous flow of water appeared adequate to eliminate accumulation of released metabolites, as judged from the absence of a drop of pH and sulphide in the incubation water (Fig. 5A, B). However, we observed that this had only a small effect on anoxic survival time, LT_{50} increasing from 8.3 to 9.0 days ($P < 0.01$). This is in sharp contrast to the positive effect the addition of chloramphenicol displayed, in both the static and the flow-through systems. In the static system, LT_{50} increased from 8.3 to 17.9 days, and in the flow-through system from 9.0 to 23.0 days. These data are in accordance with our previous results in which survival of four bivalve species from the Adriatic Sea increased two- to fourfold by the addition of chloramphenicol. Survival time was not enhanced when the medium was exchanged daily (semi-static incubation). In the latter experiment, increasing levels of sulphide were detected in the incubation medium, which indicated that the responsible microorganisms were attached to the clams and not introduced with the seawater (de Zwaan et al. 2001a). The same accumulation pattern was observed for *M. balthica* in the present experiment, although the bivalve species came from very different ecosystems. Both studies indicate that anoxia induced the growth of sulphate-reducing bacteria, which remain associated with the bivalves even when water was exchanged daily (semi-static) or continuously (flow-through). Overall, the evidence strongly indicates that bacteria disrupted the biology of the bivalves, leading eventually to death.

The fact that survival time in the static and in the flow-through system was almost identical indicates that the noxious effect of bacteria is not caused by the accumulation of volatile or soluble metabolites but by proliferation of bacteria, possibly inside the shells.

In the presence of chloramphenicol, the 28% lower survival time in the static as compared to the flow-through system might be caused by the release of volatile fatty acids, well-documented fermentation products of bivalves during long-term anaerobiosis (de Zwaan and Mathieu 1992). The volatile acids (propionate and acetate) may have served as substrate of a small group of chloramphenicol-resistant bacteria that appear after prolonged anaerobic incubation. Increased mortality was not due to the decrease in pH caused by these acids (Fig. 4B). When the pH was stabilised by Tris-HCl buffer this proved disadvantageous to the clams (Fig. 6A; Table 3). In the presence of chloramphenicol, the pH effect was smaller. This indicates that slight acidification reduces proliferation of bacteria, which is in accordance with lower biotic sulphide release (Fig. 6B).

In the blood clam *Scapharca inaequivalvis* chloramphenicol was able to decrease the accumulation of sulphide considerably, but accumulation finally took place in the presence of the antibiotic. Seemingly protected by the clam, a significant population of sulphate-reducing bacteria was able to survive in the presence of the antibiotic (de Zwaan et al. 2001a). Preliminary studies by A. Hernandez Palomino, in collaboration with the first author, showed that sulphide-oxidising and sulphate-reducing bacteria occur not only externally associated with an apparently healthy blood clam, but also appear to coinhabit different tissues, even in the blood system. In *M. balthica* virtually no sulphide accumulated in the presence of chloramphenicol (Fig. 4A). This indicates that the bacteria were not protected against the antibiotic by the bivalve. For the most part, they probably were superficially attached to outer tissues, such as the shell and the tissues lining the extrapallial cavity (gills and mantle epithelium). This may also explain why survival time in air was substantially longer as compared to in anoxic seawater (Fig. 3). In air O_2 might have prevented the growth of bacteria like the antibiotic did in anoxic water. This assumption is further supported by the fact that survival time in an atmosphere of N_2 was comparable to that in anoxic seawater (Fig. 6A). During emersion, clams apparently kept their shell valves closed until death. Aerial oxygen consumption, a well-known feature for some bivalves (Widdows and Shick 1985), probably was of minor importance. Emersion in air reduces or blocks the uptake and distribution of oxygen by

the gills and haemolymph of the bivalve, which switches to anoxic metabolism, whereas aerial oxygen has full access to the superficially attached bacteria. This condition resembles the oxic (seawater > 50% air saturation)-sulphidic incubations of populations of *M. balthica* by Jahn and Theede (1997). Sulphide may diffuse into the mitochondrion and evoke tissue anoxia by blocking cytochrome aa_3. No differences were observed by the authors in lethal times under anoxic or anoxic-sulphidic conditions (they did not check for biotic sulphide formation in the anoxic condition). In the oxic-sulphidic incubations, populations from low-sulphide-contaminated environments survived the same period. However, clams collected from sulphidic habitats survived under these conditions about two to five times longer. The authors found that the populations from a high-sulphide habitat apparently had sulphide-diffusion coefficients which were roughly half those of sulphide-sensitive specimens. In consequence, they assumed that the lower mortality rate of the former population was due to better protection of cytochrome *c* oxidase against sulphide toxication, thus resulting in their energy metabolism not being entirely based on anaerobic metabolism. Unfortunately, no determinations of fermentative products in the clams were carried out. Moreover, the authors also established that the inhibition constant of cytochrome *c* oxidase for sulphide from the sulphide-tolerant populations was about threefold lower than that of the other populations. Corresponding with the salinity at the collection site, the sulphide-adapted species were incubated at 9‰ and the intolerant ones at 22‰ salinity. The possibility that sulphide-tolerant populations of *M. balthica* survived two- to threefold longer under oxic-sulphuric conditions, because bacterial growth was prevented due to the oxygenated water in combination with low salinity (9‰), needs to be considered as an alternative explanation.

In conclusion, our studies strongly indicate that bacterial attachment on bivalves interferes strongly with survival time under experimental anoxic conditions. The adverse effects were to a minor extent caused by water quality deterioration due to release and accumulation (static system) of noxious metabolic wastes. The major cause , however, appears to be that experimental anoxia enabled bacteria to proliferate, likely causing direct bacterial infection of the bivalve. This explains why regular exchange (de Zwaan et al. 2001a), or the use of a constant flow of incubation medium (present study), hardly increased survival time. The defence mechanisms of the stressed bivalve probably could not handle the situation any longer, and eventually bacteria disintegrated the body. In accordance, the addition of chloramphenicol strongly increased survival time, independent of the incubation system employed.

Since bacterial outbreaks are a part of every benthic anoxic event, the bacterial interference observed may also have great impact on anoxic survival of bivalves, and other invertebrates, in their habitat. A number of biochemical adaptations have been described that enable survival of bivalves under environmental anoxia. These include, on the one hand, the maintenance of large stores of fermentable fuels in all tissues and, on the other hand, mechanisms for minimising ATP utilisation rate (de Zwaan and Mathieu 1992). Our findings that bacterial infection is likely the cause of death under severe hypoxia challenge the importance of biochemical adaptations in the field. Recently we observed that 50% of cockles (*Cerastoderma edule*) incubated in anoxic seawater died after 3.5 days, still with half the initial glycogen concentration present in the tissues. However, in the presence of chloramphenicol (LT_{50} 7.9 days), the cockles utilised their endogenous fuel almost completely (Babarro and de Zwaan 2001).

Acknowledgements The authors would like to thank R. Bogaards for technical assistance and help with the harvest of animals. We are also grateful to A. Ernst for the critical review of the manuscript. J. M. F. Babarro acknowledges the receipt of a post-doctoral fellowship from the Ministerio de Educacion y Ciencia (Spain, 1999–2001). This is publication no. 2632 of NIOO-CEMO. The authors would like to acknowledge an anonymous reviewer for his important suggestions to improve the manuscript.

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