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Effect of H_2S on heterotrophic substrate uptake, extracellular enzyme activity and growth of brackish water bacteria

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ABSTRACT: In some parts of the Baltic Sea, establishment of anoxia, H_2S accumulation and reaeration of the water column are common processes which have a bearing on the heterotrophic microbial community. The influence of these conditions on natural aerobic bacteria populations was studied in 24 h batch cultures. Measured parameters were: saprophyte number and total number of bacteria, active bacteria (leucine microautoradiography), heterotrophic substrate uptake (^{14}C labelled glucose, leucine, lactate) extracellular enzyme activity (β-glucosidase, peptidase) and growth of bacteria (^{3}H-methyl thymidine incorporation). Anoxia established by N_2 had a minor effect on these parameters and values approximated those from the oxic control within the incubation period. Addition of H_2S led immediately to a strong but variable reduction in all the activity measurements and recovery was weak when H_2S conditions were maintained over the experimental period. Re-aeration after 12 h of H_2S incubation caused a progressive increase of the activity measurements which then by far exceeded those from the continuously oxic control. An exception was peptidase, which recovered only slowly after H_2S depletion. Short-term application of H_2S caused clear changes in the metabolic and community structure of the originally aerobic bacterial population, which were also documented by a reduction of the spectrum of morphological cell characters. Cells which survived H_2S stress developed vigorously after H_2S depletion. Values of bacterial production calculated from increases in active bacterial numbers and from thymidine uptake showed the same tendency; however, their absolute values differed considerably. This discrepancy may indicate that after H_2S stress many of the surviving cells were reactivated, but only a fraction of these started reproduction.

INTRODUCTION

Considerable parts of the deeper zones of the Baltic Proper and also some isolated deep parts and trenches in coastal areas are permanently or temporarily depleted of oxygen. As a result of organic nutrient accumulation and stratification by a permanent halocline, microbial sulfate reduction may lead to H_2S accumulation in restricted zones within these areas. This process starts from the sediments, mostly during late summer (Bansemir & Rheinheimer 1974). H_2S conditions may be stable throughout the year, as in some of the deep basins, e.g. Gotland Deep, Farö Deep, or unstable due to the specific hydrographic regime in the shallow water fjords of the Baltic Sea (Ehrhardt & Wenck 1984). Microbes in the chemocline may be subject to changing redox conditions, because the position of the chemocline in the water column is variable (Rheinheimer et al. 1989).

These environmental events can be expected to be accompanied by drastic changes in the microbial community (Gast & Gocke 1988) and its biochemical activity, which in turn will have an influence on the living biota and water chemistry. The effect of H_2S on microbial populations may be studied in 2 contexts: (1) changes which occur when oxic waters are submitted to H_2S, and (2) changes which occur when anoxic, H_2S-containing waters together with their adapted microbial population are submitted to oxic conditions. In the present investigation the first aspect was studied in laboratory experiments.

Since the establishment of anoxic environments in the Baltic Sea is mostly a consequence of microbial

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organic matter degradation, parameters investigated were closely related to heterotrophic properties of the bacterial population. Processes of chemoautotrophy inherent in oxygen depletion and the presence of H_2S have been described in detail elsewhere (e.g. Fenchel & Blackburn 1979). Our interest was focussed on the influence of H_2S on heterotrophic substrate uptake and extracellular enzyme activity and their relationship to microbial biomass and production patterns. The laboratory experiments were conducted for about 24 h; thus only the initial effects of H_2S on microbial populations and no effects of long-term adaptation were studied. It is, however, most likely that in many regions of the Baltic Sea, especially in the water layer above the sediment, conditions rapid change from oxic to anoxic and vice versa.

In our experiments we attempted to simulate a natural process which cannot easily be followed in the natural environment, since it occurs at considerable depth and would require repeated sampling of exactly the same small body of water. The laboratory experiments presented were stimulated by field observations in the Gotland Deep of the Baltic, where a dramatic gradient of bacterial abundance was found in the transient layer between oxic and anoxic water masses (Gast & Gocke 1988, Rheinheimer et al. 1989). Further, in the oxic/anoxic interface of a Norwegian estuary, total bacteria numbers were higher than in surrounding waters (Indrebø et al. 1979). Contrary observations were made by Hobson (1983) and Juniper & Brinkhurst (1986), who did not find enhancement of microbial heterotrophic activity in the oxic/anoxic interface of the Saanich Inlet (British Columbia, Canada).

The experiments were generally conducted with oxic brackish water from the Kiel Fjord. An experiment with water from a stratified lake is also included, to provide some information on whether the influence of H_2S on brackish water and fresh water bacterial populations is principally the same.

MATERIALS AND METHODS

Four batch culture experiments were performed, 3 with water from the Kiel Fjord and 1 with water from Lake Plußsee near Plön (Northern Germany). For each experiment 8 l aliquots of surface water (1 m) were transferred to three 10 l Duran-glass experimental vessels (Batches 1 to 3). In all experiments the *first vessel* served as an oxic control and was continuously aerated with sterile air The treatment of the *third vessel* was also the same in all experiments: it was deoxygenated with N_2 for 30 min and then supplemented with Na_2S, resulting in sulfide concentrations of 20 to 30 µM. In Expts 1 and 2 the *second vessel* was continuously bubbled with N_2 leading to almost undetectable concentrations of O_2. In Expts 3 and 4 the *second vessel* was treated as described already for the third vessel. After 12 h, however, it was reaerated by bubbling with sterile air. Subsamples for measurements were taken after 0, 6, 12, 18 and 24 h of incubation, taking care not to alter the oxic/anoxic conditions. The water in the vessels was agitated by a magnetic stirrer and held at 20 °C.

The measured variables were: total numbers and biomasses of bacteria, by acridine orange staining and epifluorescence microscopy (Zimmermann 1977); saprophyte numbers, on ZoBell-agar; heterotrophic uptake of ^{14}C labelled glucose, lactate, acetate and leucine (Gocke 1977); microbial extracellular enzyme activity of peptidases and α/β-glucosidases, as measured by means of fluorogenic model substrates (Hoppe 1983, Hoppe et al. 1988); bacterial production, as measured by means of ^{3}H-methyl thymidine incorporation (Fuhrman & Azam 1982); numbers of actively metabolizing bacteria, by microautoradiography with leucine as a substrate (Hoppe 1976); and oxygen and H_2S concentrations, by means of standard procedures.

At each sampling time, 3 replicate measurements were made for heterotrophic substrate uptake, ^{3}H-methyl thymidine incorporation and plate counts; for the other measurements no replicates could be made. Incubations of the subsamples were carried out at 20 °C for 3 h for heterotrophic substrate uptake, ^{3}H-methyl thymidine incorporation and microautoradiography and about 4 h for extracellular enzyme activity determinations. The subsamples were kept exactly at the same oxic, anoxic or H_2S-conditions as they were in the vessels from which they originated.

For anoxic incubations all liquid reagents needed for the different procedures (e.g. radiotracers, fluorogenic model-substrates) were made oxygen-free by bubbling with N_2. Incubations for anoxic extracellular enzyme activity measurements were made in plastic syringes which were flushed with N_2 before use. The substrate was then introduced through the socket for the needle and the needle was fixed to the syringe. Then the samples from the experimental vessels were directly sucked into the syringe through the needle. Finally the needle was closed by a rubber plug.

Anoxic subsamplings for heterotrophic substrate uptake measurements and bacterial production estimates were done according to the filling procedure of BOD-bottles. For this, water was released from the hermetically closed experimental vessels by N_2 gas pressure. The required amount of radioactive liquid was then introduced into completely filled incubation bottles by means of a syringe equipped with a long needle.

Anoxic incubations for subsequent autoradiography

were performed in 2 ml test-tubes. The tubes were completely filled in the manner as described before and stoppered with a capillary-fitted rubber plug. The radioactive substrate (10 µCi ^{3}H-leucine per ml sample) was then introduced through this capillary which was fitted with a short silicon tube, so that surplus liquid could rise from the test-tube. Finally the silicon tube was closed with a small steel plug.

Results were expressed in terms of bacteria ml^{-1} and bacterial biomass l^{-1} (= µg C l^{-1}). For heterotrophic activity maximum uptake velocity (V_{max} = µg C $l^{-1}h^{-1}$) and turnover rate (T_R = % h^{-1}), and for extracellular enzyme activity maximum hydrolysis velocity (V_{mh} = µg C $l^{-1}h^{-1}$) and turnover time (T_t = h) were calculated. Bacterial production is presented as increase of bacterial carbon (BP = µg C $l^{-1}h^{-1}$). Factors used to convert thymidine incorporation to bacterial production were 1.1×10^9 cells $nmol^{-1}$ (Riemann et al. 1987), 3.5×10^{-7} µg C μm^{-3} (Bjørnsen 1986) and 0.054 μm^3 measured as a mean cell volume. Bacterial production on the basis of active bacteria (microautoradiography) was calculated by using increase of active bacteria counts per unit time.

RESULTS

The first 2 experiments were run, respectively, with surface brackish water from the Kiel Fjord (March 1983) and Lake Plußsee (May 1983). Conditions of incubation were oxic, anoxic with nitrogen and anoxic with H_2S. Subsamples for analysis were taken after 1 h of conditioning and after 24 h. Results of these experiments are presented in Tables 1 and 2.

Table 1. Expt 1 Effect of anoxic conditions on natural bacterial populations from oxic brackish water (Kiel Fjord), immediately after exposure and after 24 h of incubation

Parameter	Batch 1 (oxic)		Batch 2 (anoxic; N_2)		Batch 3 (anoxic; H_2S)	
	0 h	24 h	0 h	24 h	0 h	24 h
Saprophytes[a]	5700	22 400	7240	40 600	6400	14 920
AODC ($\times 10^6$)[a]	3.06	4.59	2.91	4.28	2.84	2.31
Active bacteria ($\times 10^3$)[a]	143	622	20	575	6	2.6
V_m (glucose)[b]	0.54	2.03	0.29	2.23	0.01	0.03
% mineralization	27.4	31.3	36.9	30.4	39.3	27.8
Peptidase[c]	2.17	3.37	2.15	3.85	0.81	0.67
α-Glucosidase[c]	0.62	0.90	0.48	1.25	0.13	0.18
β-Glucosidase[c]	0.55	0.88	0.39	1.13	0.18	0.19
N-acetyl-glucosaminidase[c]	0.53	0.59	0.39	0.65	0.37	0.39
Phosphatase[c]	1.57	1.04	1.89	1.67	1.38	0.87

[a] Bacteria numbers ml^{-1}
[b] µg $C_{glucose}$ l^{-1} h^{-1}
[c] Relative fluorescence units, increase of fluorescence h^{-1}

Table 2. Expt 2. Effect of anoxic conditions on natural bacterial populations from oxic fresh water (Lake Plußsee), immediately after exposure and after 24 h of incubation

Parameter	Batch 1 (oxic)		Batch 2 (anoxic; N_2)		Batch 3 (anoxic; H_2S)	
	0 h	24 h	0 h	24 h	0 h	24 h
Saprophytes[a]	788	1562	954	1498	974	2892
AODC ($\times 10^6$)[a]	20.3	28.7	18.3	21.4	16.7	17.8
Active bacteria ($\times 10^3$)[a]	619	–	320	450	3.4	22.1
V_m (glucose)[b]	0.61	0.94	0.59	0.87	0.006	0.056
% mineralization	33.0	31.2	36.2	30.3	38.5	29.4
Peptidase[c]	5.38	5.42	5.13	5.54	1.55	1.20
α-Glucosidase[c]	0.79	0.81	0.70	0.64	0.25	0.23
β-Glucosidase[c]	0.79	0.84	0.76	0.70	0.29	0.29
N-acetyl-glucosaminidase[c]	0.61	0.64	0.54	0.54	0.23	0.21
Phosphatase[c]	2.56	2.87	2.41	3.13	1.99	1.75

[a] Bacteria numbers ml^{-1}
[b] µg $C_{glucose}$ l^{-1} h^{-1}
[c] Fluorescence units, increase of fluorescence h^{-1}

Experiment 1: anoxia and H_2S in brackish water

In the fjord experiment saprophyte numbers were similar in the 3 batches after 1 h of conditioning. They increased considerably after 24 h; in the H_2S batch, however, the increase was only 51 % of that found in the oxic control (Table 1). Total numbers of bacteria were also very similar at the beginning; after 24 h they increased considerably in the oxic and anoxic (nitrogen) batchers. In the H_2S batch they decreased, being now only 50 % of the oxic control. With respect to microbial numbers and activity the influence of anoxic conditions could be monitored best by microautoradiography. In the oxic batch after 1 h of incubation 4.7 % of the total bacteria number was metabolically active. After 24 h this increased to 13.5 %. In the batch which was made anoxic with N_2, initially only 0.7 % of the cells were active, whereas after 24 h as many as 13.4 % – almost as many as in the oxic control – were active. This indicates an adaptation of the bacteria to anoxic conditions. In the H_2S batch the ratio between total bacteria number and metabolic active bacteria dropped drastically to 0.2 and 0.1 % after 1 and 24 h of incubation, respectively.

Heterotrophic glucose uptake (V_m) in the 3 batches was closely related to the findings with the active bacteria. V_m of glucose in the N_2 batch was initially about half that of the oxic control and was nearly negligible in the H_2S batch. After 24 h of incubation glucose uptake in the N_2 batch adjusted to the oxic control (as also found for the active bacteria number). In the H_2S batch is recovered very slightly. Turnover rates of glucose (not shown in the table) were significantly correlated with uptake velocities. They varied between 3 and 9 % h^{-1} in the oxic and the N_2 batch; in the H_2S-batch they were extremely low (0.1 to 0.2 % h^{-1}). Extracellular enzyme activities were also reduced by H_2S; however to a much lesser degree than the uptake process. Compared with the values from the oxic control, peptidase as well as α- and β-glucosidase, activities in the H_2S batch were about 20 to 37 %, whereas glucosaminidases (chitinases) were about 70 % and phosphatases 85 to 90 %. In the batch which was made anoxic with N_2 only minor reductions occurred after 1 h of incubation; after 24 h extracellular enzyme activities fully recovered or even exceeded the values from the oxic control.

Experiment 2: anoxia and H_2S in fresh water

The second experiment was done with fresh water from Lake Plußsee in order to test whether substantial differences exist between the response of brackish and fresh water bacterial populations towards anoxic conditions. The experiment was therefore designed exactly as that described above. In contrast to the findings with brackish water, the increase of saprophyte counts after 24 h in the H_2S batch compared with the oxic batch was not reduced (Table 2). Total numbers of bacteria did not show such a strong increase in the oxic and N_2 anoxic batch after 24 h as was observed in brackish water. In the H_2S batch they increased slightly, while they decreased in the brackish water experiment. Numbers of actively metabolizing bacteria in fresh water were initially very strongly affected by H_2S conditions: they decreased to 0.02 % of total numbers, but after 24 h recovered slightly, to 0.1 % of total bacterial numbers. Heterotrophic glucose uptake values, compared to the total numbers of bacteria, were much lower in freshwater than in brackish water. H_2S had a very strong effect on glucose uptake, as already indicated by the numbers of active bacteria. In fresh water, as for brackish water, the effect of oxygen depletion by N_2 was low on all the extracellular enzyme activities measured. In fresh water H_2S also caused a strong reduction in substrate hydrolysis. However, this reduction was somewhat less for α- and β-glucosidases. Glucosaminidases (chitinases), on the other hand, were reduced much more strongly (about 35 % of the value from the oxic batch) than in brackish water.

Experiment 3: anoxia, H_2S and reaeration in brackish water

The last 2 experiments were again carried out with brackish water from the Kiel Fjord. In this case, the effect of reaeration on bacteria exposed to H_2S conditions was also investigated. Therefore, in addition to the oxic control, two H_2S batches were run, one of which was reaerated after 12 h of H_2S incubation.

The third experiment (first reaeration experiment) was run in October 1985. The oxic and the continuously anoxic H_2S batches showed nearly the same development with respect to extracellular enzyme activities and glucose uptake as in the first brackish water experiment (Fig. 1). This was also true for the second H_2S batch before reaeration. Only the reduction of β-glucosidase activity in the H_2S batches was generally somewhat lower than in the first brackish water experiment. Reaeration of the second H_2S batch after 12 h caused only a weak increase and slow recovery of extracellular enzymes. In contrast to this, glucose uptake rapidly increased after reaeration, exceeding by far the values from the oxic control. Later on glucose uptake in the reaerated batch adjusted to the oxic control. Lactate, which is believed to play a major role in anoxic environments, showed in principle the same uptake pattern as found for glucose, however there

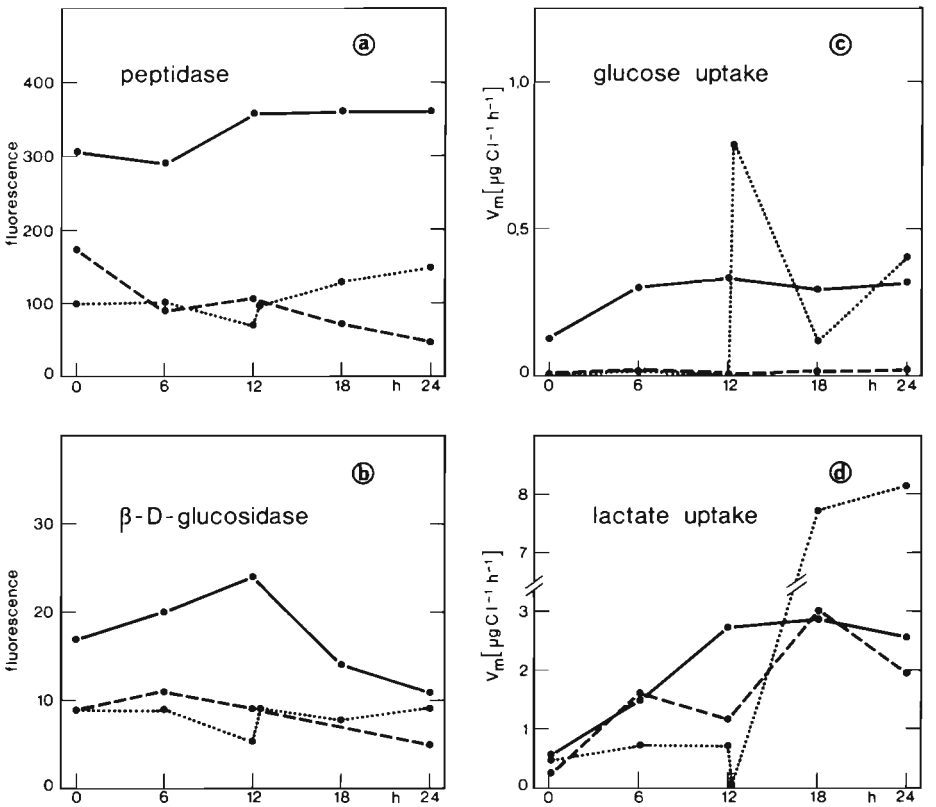

Fig. 1. Activities of (a, b) extracellular enzymes and (c, d) substrate uptake of brackish water bacteria in batch cultures; results from the first reaeration experiment (Expt 3). (●——●) oxic conditions; (●·····●) H_2S conditions for 12 h, then reaeration and immediate subsampling; (●– – –●) H_2S conditions for 24 h

were some specific deviations. Lactate uptake was far less inhibited by H_2S conditions than glucose uptake, which was nearly completely reduced. Reaeration of short-time H_2S-adapted bacteria populations caused at first a decrease of lactate uptake and then an extremely strong increase.

Experiment 4: anoxia, H_2S and reaeration in brackish water

The fourth experiment (second re-aeration experiment) (October 1987) was designed as Expt 3. However, for the first time bacterial production measurements were also included. H_2S was not renewed in the 2 anoxic batches (renewal always proved to be necessary for the maintainance of stable H_2S conditions) and thus it slowly disappeared from them. Nevertheless anoxic conditions were maintained by the N_2 atmosphere in one of the H_2S vessels. Therefore the results of this experiment give insight both into slow readaptation of originally aerobic bacteria populations after exposure to H_2S, and also into the effects of pulse introduction of air, as occurred in the reaerated H_2S batch.

Total bacteria numbers, in this experiment, behaved rather conservatively in the 3 batches (Table 3). During the 24 h incubation period in the oxic control, numbers remained nearly the same: there was a decrease of about 10 % in the continuously anoxic batch and of about 20 % in the reaerated batch.

The numbers of actively metabolizing bacteria were initially reduced in the anoxic batches. The reduction, however, was not as pronounced as found in the first brackish water experiment. In the oxic batch, an increase of 2.4-fold during the 24 h incubation was observed (Fig. 2b), though some oscillation in the values occurred. This oscillation was most likely due to flagellate grazing as many of these were occasionally observed on the autoradiographic slides. In the continuously anoxic and the reaerated batches the curves for active bacteria were rather similar. After 12 h of incubation numbers of active bacteria increased rapidly, becoming much higher than those from the oxic control. Obviously, after the H_2S period bacteria activity was stimulated simultaneously in the pulse reaerated vessel and also in the vessel where H_2S decreased continuously. This is indicated by the strong increase of the percent of active bacteria and active bacterial biomass of total bacteria and total bacteria biomass after 24 h (Table 3).

The findings with the metabolically active bacteria were reflected by the heterotrophic substrate uptake measurements in the 3 experimental vessels (Fig. 3). In principal the uptake of the 2 substrates used for the experiments, glucose and leucine, behaved in the same

Table 3. Expt 4. Results from the second reaeration experiment. Only measurements from the beginning and the end of the experiment are given. Note strong increases of % of active bacteria and active bacteria biomass of total bacteria number and total bacteria biomass after 24 h. Active bacteria biomass was calculated on the assumption that mean carbon contents of active and total bacteria cells are similar

Batch no.	Conditions	Tot. bact. (no. ml^{-1})	Act. bact. (no. ml^{-1})	% Act. bact.	Tot. bact. biomass ($\mu g\ C\ l^{-1}$)	Approx. act. biomass ($\mu g\ C\ l^{-1}$)	Bact. prod. ($\mu g\ C\ l^{-1}\ h^{-1}$)
Results immediately after establishment of indicated conditions							
1	Oxic	4.85×10^6	0.35×10^6	7.2	92.4	6.7	0.158
2	H_2S	5.31×10^6	0.24×10^6	4.5	101.1	4.6	0.035
3	H_2S	5.34×10^6	0.24×10^6	4.5	101.8	4.6	0.034
Results after 24 h under indicated conditions							
1	Oxic	4.58×10^6	0.84×10^6	18.3	44.7	8.2	0.338
2	Reaerated after 12 h	4.08×10^6	1.61×10^6	39.5	65.7	25.9	0.758
3	Anoxic without H_2S	4.87×10^6	2.13×10^6	43.7	92.8	40.6	0.943

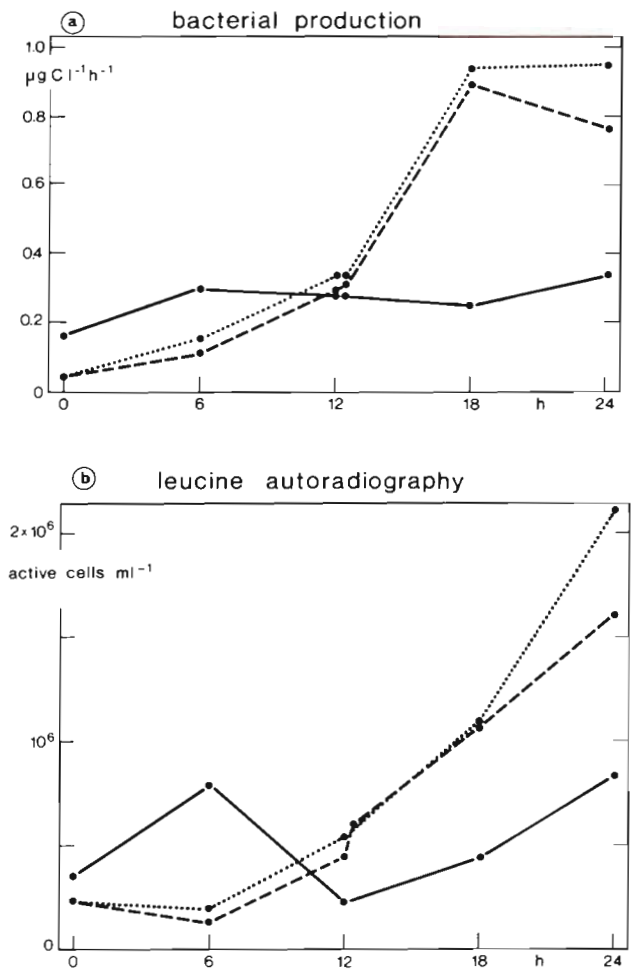

Fig. 2. Results of bacteria production derived from ^{3}H-methyl-thymidine incorporation (a) and of active bacteria numbers derived from microautoradiography (b) from the second re-aeration experiment (Expt 4). Conditions were oxic (●——●), H_2S-anoxic and reaerated after 12 h (●– –●) and H_2S/N_2 anoxic (●·····●)

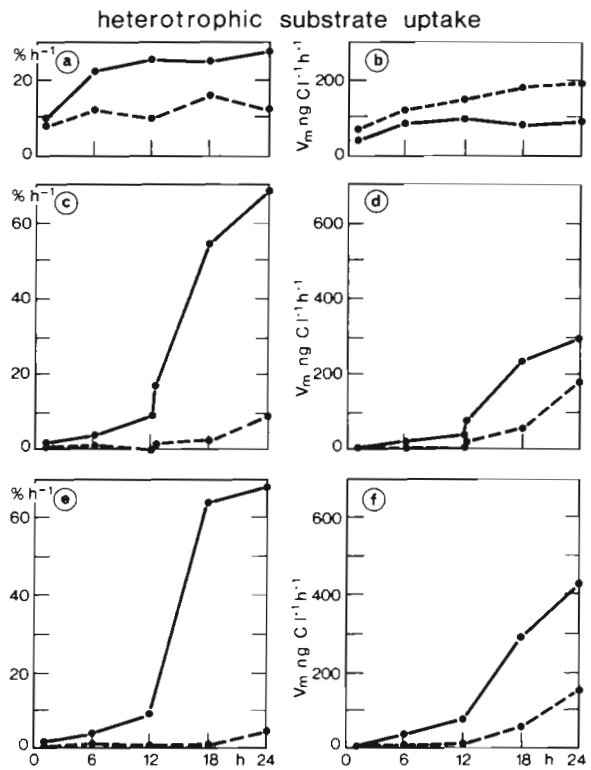

Fig. 3. Results of heterotrophic substate uptake (% h^{-1} = turnover rate and V_m) for glucose (●–●) and leucine (●——●)from the second re-aeration experiment (Expt 4). Conditions were (a, b) oxic, H_2S-anoxic, (c, d) re-aerated after 12 h, and (e, f) H_2S/N_2 anoxic

way, showing initially a strong inhibition after application of H_2S and a strong increase when the influence of H_2S ceased or reaeration occurred. However, V_m values reveal that there was a preference for glucose uptake in the oxic vessel, while in the anoxic ones leucine uptake dominated throughout the experiment. Turnover rates

of both substrates were extremely low during the H_2S period of incubation. They recovered very strongly for leucine uptake after the H_2S period, exceeding by far the values of the oxic control. Values for glucose turnover recovered only slowly.

The results from the ^{3}H-thymidine incorporation measurements (Fig. 2a) are in line with those obtained from autoradiography and heterotrophic substrate uptake. Absolute values of bacteria production were initially low (0.16 µg C $l^{-1}h^{-1}$ in the oxic batch; 0.035 in the anoxic batches) which corresponds with the low values for heterotrophic substrate uptake and active bacteria numbers. After 12 h of incubation bacteria production in the reaerated batch and also in the continuously anoxic batch exceeded by far that in the oxic control, again the same tendency as for active bacteria and leucine uptake. After 24 h of incubation, bacteria production was 0.34 µg C $l^{-1}h^{-1}$ in the oxic batch, 0.76 in the anoxic batch and 0.94 in the reaerated batch, respectively (Table 3).

The extracellular peptidase activity measurements deviated from these observations (Fig. 4a). Peptidase activities in the oxic control were always above those found in the anoxic batches, whether they were re-aerated or not. Re-aeration in one of the H_2S batches after 12 h caused a progressive increase in peptidase activities. In the other batch, where H_2S slowly disappeared but anoxic conditions were still maintained by N_2, peptidase activities did not recover during the incubation time. This is clearly different from the findings with the other activity parameters measured, and will be discussed below. The results from V_m of peptidases are reflected by the turnover time of peptides in the water (Fig. 4b). Turnover times in the anoxic batches were always much longer than those from the oxic control. After reaeration they decreased and approached the oxic control. In the batch where H_2S deplition occurred, turnover times also decreased after 12 h; this is certainly for other reasons than the decrease in the reaerated batch, because the V_m in this batch did not shift adequately.

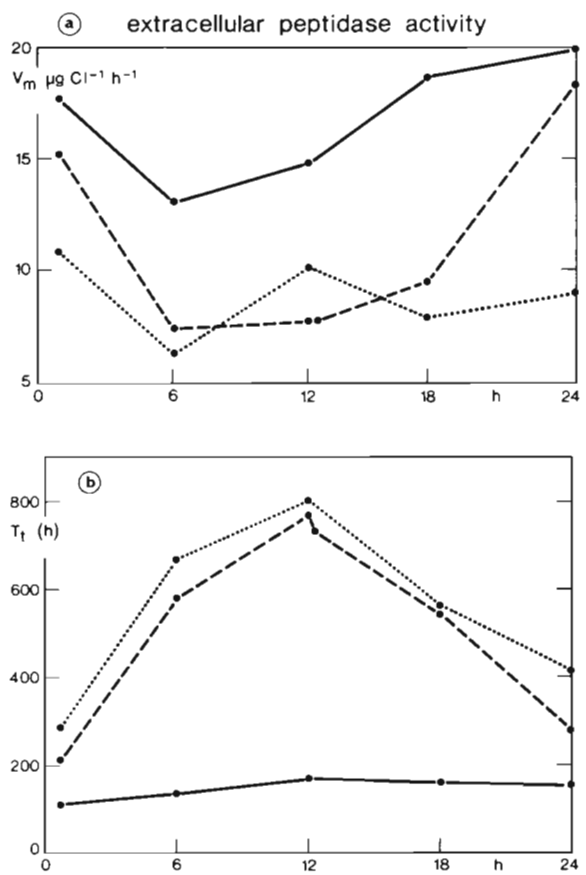

Fig. 4. Results of peptidase activities (V_m, turnover time) from the second reaeration experiment (Expt 4). Conditions were oxic (●—●), H_2S-anoxic and reaerated after 12 h (●–●) and H_2S/N_2 anoxic (●···●)

DISCUSSION

The initial response of aerobic microbial populations from sea- and lakewater towards H_2S was investigated. It is expected that no obligate anaerobic bacteria were among these populations though it is known that some sulfate-reducing bacteria can withstand oxygen stress for more than 3 h (Cypionka et al. 1985). However, the samples for the laboratory experiments were taken from surface water and had certainly had no contact with anoxic water for a considerable period of time. There may have been some facultatively anaerobic denitrifiers present, which switched over from O_2 to nitrate-respiration during experimental H_2S incubation (Rheinheimer 1985). The abundance of these microorganisms in well-aerated marine surface waters in comparison to the total bacteria number is believed to be low. On the other hand there was sufficient nitrate (18 µmol l^{-1}) present in the water for these organisms to develop under anoxic conditions, as long as they were not negatively affected by H_2S. Chemoautotrophic bacteria may have been included in the total counts but less so in the quantitative activity measurements of our experiments, because obligately autotrophic bacteria, at least, may not take up organic solutes, e.g. thymidine (Johnstone & Jones 1989). Also these bacteria are not abundant in seawater. From these considerations about the natural microbial community used in our experiments we conclude that the measured responses towards anoxia and H_2S were mainly due to the prevailing aerobic heterotrophic microbial population.

The results of the different measurements from the 4 experiments show similar tendencies of which the most pronounced is the initial reduction of microbial

activities after establishment of H_2S conditions and the subsequent recovery or stimulation of growth by re-aeration. With respect to bacteria numbers, substrate uptake and enzyme activity, the aerobic natural bacteria populations were not strongly influenced by anaerobic or microaerobic conditions as established by flushing with N_2. Exposure to H_2S, however, led immediately to a strong reduction of all these parameters, and also of bacterial production, except total numbers of bacteria, where a distinction between living and dead or inactivated cells is not get possible. However, the degree of reduction for the different measurements varied considerably.

Influence of H_2S on substrate uptake

Heterotrophic substrate uptake velocities of glucose and leucine as well as their turnover rates were reduced strongly at the beginning of the H_2S influence and recovered only very slowly after 24 h under persisting H_2S conditions. Lactate uptake was less influenced by these conditions, as this substrate is possibly more influenced by anaerobic metabolism of facultatively anaerobic bacteria in the water. Thus it seems that the behaviour of heterotrophic substrate uptake under these conditions is also a function of the substrate in question. Metabolically active bacteria numbers traced by ^3H-leucine microautoradiography decreased drastically in the H_2S batches and there was also a reduction of the original diversity of morphological cell characters. This agreed well with heterotrophic leucine and glucose incorporation, however it did not reflect the high capacity for lactate incorporation. It could be that the few remaining active bacteria had very high individual lactate uptake rates or that leucine, used as a substrate for active bacteria detection, was not adequate for the substrate demands of bacteria developing in anoxic (H_2S) waters. Previous experiments on acetate uptake revealed that a very low number of acetate-utilizing bacteria (5 to 20 % of the total active bacteria population; Hoppe 1978) caused very high uptake rates, resulting in extremely large spots on the X-ray film.

Influence of H_2S and reaeration on extracellular enzyme activity

Another interesting point is the different behaviour of bacterial substrate uptake and extracellular enzyme activity towards H_2S. The minor decrease of the latter suggests that exoenzymes are only partly inhibited by H_2S, probably preferentially the heavy metal containing enzymes (e.g. metalloproteases) and others which tend to establish disulfide bonds (Forth et al. 1984). This fraction is only slowly reactivated or re-established after expulsion of H_2S by air. The remaining part retains its activity independently of the O_2/H_2S regime. However, the relationship between the active and the inactivated fraction varies with the different enzymes tested. In principle, enzymes from brackish water and freshwater populations were affected by H_2S in the same range of inhibition. Only N-acetyl-glucosaminidase and phosphatase were comparatively much less inhibited in brackish water from the Kiel Fjord (Tables 1 and 2), a difference which cannot be readily explained.

Similar experiments were done by Reichardt (1986) with bacteria from brackish water sediments. It was found that protease activity on particulate protein model substrates (hyde powder azure) decreased drastically in the reduced zone of the sediment. Activities there were about 21 % of those found in the upper oxic zone. Chitinase activities were not affected by Eh of the sediments. Oxygen depletion in undisturbed oxic sediments led also to a decrease in proteolytic activity. In homogenized sediment samples there was no response to aeration or O_2-depletion. This leads to the conclusion that there is a close relation between the microbial proteases and the physiological state of their producers. Daatselaar & Harder (1974) found that the growth rate is an important parameter for the control of the synthesis of extracellular proteases. Pore water analysis (Rosso & Azam 1987) also revealed a 50 % decrease of the proteolytic activity in marine sediment in comparison to the water sediment interface. Unfortunately no data on Eh or oxygen concentration in the sediment or on the conditions of incubation were supplied.

Uptake systems for some important organic nutrients are obviously nearly totally inhibited by H_2S, however they recover immediately after reaeration, if H_2S is applied only for short periods. The uptake systems of bacteria operate less independently than the extracellular enzymes, which are located outside the cell membrane and which are not, or not as, closely coupled with the cell metabolism (Priest 1984). The uptake systems are energy dependent and related to metabolic reactions in the cell (Krambeck 1979); thus their inhibition may be directly affected by H_2S or indirectly, e.g. when other cell functions (such as respiration) are affected.

Effect of H_2S and reaeration on bacteria production

Under exposure to H_2S, thymidine incorporation into bacterial DNA, used as a measurement of bacterial production, decreased to a lesser extent than uptake of glucose and leucine. Of course it cannot be expected

that this parameter would react immediately on nutrient cut off, because internal storage pools may be used for further synthesis for a probably short period of time. In addition, the use of other types of substrates than glucose and leucine, e.g. lactate and/or acetate, may also be responsible for relatively high DNA-synthesis under anoxic conditions. The question of thymidine incorporation by microbes from anoxic waters has been discussed by Pollard & Moriarty (1984) and McDonough et al. (1986). It was pointed out that despite their strict nutritional habits, at least some obligate anaerobic bacteria take up thymidine. McDonough et al. (1986) exposed a microbial population from an anoxic hypolimnion to oxygen and found a reduction of 66 % for thymidine incorporation into DNA and of 42 % for incorporation into protein. Our experiments revealed a strong increase (Fig. 2a) for thymidine incorporation into DNA after aeration of originally aerobic bacterial populations which were exposed to H_2S for a short time. A similar increase for thymidine incorporation was recorded in the batch where H_2S disappeared slowly but anoxic conditions were maintained by N_2. The last reading at 24 h already shows a stabilisation or even decrease of bacteria production rates, while active bacteria and leucine uptake were still increasing (Figs. 2b and 3). It is likely that when growth is approaching the stationary phase, bacteria start to allocate energy more to protein synthesis, as indicated by the leucine uptake values, than to cell devision (DNA synthesis), causing a shift in the thymidine/leucine uptake ratio (Kirchman & Hoch 1988). In the oxic batch the increase of bacteria activity and also of the active bacteria fraction was much less than in the H_2S batches. Probably due to grazing activities no clear relationships between the 3 activity parameters could be observed in this batch.

Recovery of microbial population after H_2S-stress

Disappearance of H_2S had a pronounced stimulatory effect on growth, leucine uptake and percent of active bacteria of the originally aerobic bacteria population (Figs. 2a, b and 3; Table 3). This stimulation, however, did not result in increasing total bacteria numbers and biomasses; both measurements even decreased after the 24 h experimental period (Table 3). The increase in substate uptake and growth is convincingly reflected by the increase of active bacteria and the dramatic change of the fraction of active bacteria from initially 5 % to between 40 and 44 % at the end. However, it is questionable whether this change in active bacteria numbers and biomass was due to reproduction or whether it was only a consequence of reactivation of bacteria with respect to substrate uptake after H_2S stress. The increase of active bacteria of nearly 2×10^6 ml^{-1} in the third vessel, for example, was not counterbalanced by an increase in total bacteria numbers. Because grazing can be excluded in this continously anoxic batch and cell damage is unlikely to occur during the short period of H_2S influence, an increase in total bacteria numbers should be expected if the increase in active bacteria was mainly due to reproduction. It may therefore be assumed that the increase of active bacteria numbers is partially due to reactivation of bacteria, which is documented by the strong gradient of leucine uptake. Nevertheless increasing values of thymidine incorporation suggest that reproduction also contributed to a certain extent.

On the basis of active bacteria production rate and production rates calculated from thymidine uptake, we attempted to distinguish between metabolic activation of cells and cell reproduction (Table 4). Absolute values of biomass production rates calculated from the data of

Table 4. Bacterial biomass production rates ($\mu g\ C\ l^{-1}$ per 6 h) calculated on the basis of active bacteria number increases (MAR) and thymidine incorporation (Thy). MAR production rates were calculated on the basis of active bacteria counts and average cell volume obtained from AODC preparations. Production rates on the basis of thymidine uptake were calculated from the mean Thy incorporation of the corresponding end point measurements

Incubation period	Batch 1 (oxic)		Batch 2 (H_2S re-aerated)		Batch 3 (H_2S N_2-anoxic)	
	MAR	Thy	MAR	Thy	MAR	Thy
0– 6 h	8.02	1.36	ni	0.44	ni	0.54
6–12 h	ni	1.71	6.08	1.19	6.65	1.42
12–18 h	1.27	1.56	11.78	3.53	10.26	3.83
18–24 h	3.92	1.75	10.26	4.95	19.76	5.67
Biomass increase 0–24 h	13.21	6.38	28.12	10.11	36.67	11.46

ni: no increase of active cell numbers

active bacteria number increases and thymidine incorporation were very different. This suggests that the increase of active bacteria cannot be explained only by production of bacteria cells but also by activation of resting cells in terms of substrate uptake. The 24 h production rates obtained from these 2 methodical approaches make this statement even more obvious. The ratios between the 2 production rates, calculated separately for each batch, show an increasing tendency (2.1, 2.8, 3.2), suggesting that active bacteria increases are better documented by thymidine incorporation in the oxic batch, while activation of resting cells, not necessarily combined with reproduction, is more important in the H_2S batches, especially in the third vessel, which was anoxic throughout the experimental period.

CONCLUSIONS

This investigation shows that drastic changes in bacterial metabolism occur when oxic waters are exposed to H_2S conditions; oxygen depletion alone was much less effective. The substantial differences between substrate uptake, growth and extracellular hydrolysis suggest that the balance between these parameters as it exists in oxic waters is initially decoupled in the H_2S environment. The uptake of substrates which are most important in oxic waters is probably replaced by the uptake of other substrates which are better suitable for anaerobic metabolism. DNA synthesis and extracellular hydrolysis of the bacteria behave more conservatively. Future investigations should study how these parameters stabilize after long-term adaptation to reducing conditions. Anoxic incubations with waters from anoxic zones have been mainly performed for denitrification and desulfurication measurements. McDonough et al. (1986) claim that their study of bacterial production in an anoxic hypolimnion is among the first to examine thymidine incorporation by microorganisms in anaerobic waters. More information about bacterial response to changing redox-conditions is available from sediment studies. However, direct studies with corresponding situations in the water column should be performed.

The ecological importance of boundary layers such as the oxic/anoxic interface in the Baltic Sea is clear. It has been pointed out that these boundaries may well serve as biotopes for active bacterial growth, supplying conditions for different types of energy metabolism and biomass growth as indicated by high total bacteria numbers and different uptake rates of substrates under varying environmental conditions (Rheinheimer et al. 1989). Moreover, it is likely that these boundaries also provide a favourable nutritional habitat for bacteria grazers and higher heterotrophic organisms. Observations of high numbers of flagellate bacteriovores have been made from the deeper layers of the Baltic Sea (Gocke unpubl.), suggesting the existence of a complex chemoautotrophic/heterotrophic foodweb. The existence of such a foodweb at greater depths should encourage us to focus more intensive studies on growth rates and trophic interactions as well as bacterial substrate uptake mechanisms in these environments. This would lead to a better understanding of fluxes of energy and organic matter from surface waters to the bottom layers in the Baltic Sea and other border seas where anoxic conditions occur.

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