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Nitrous oxide producing heterotrophic bacteria from the water column of the central Baltic: abundance and molecular identification

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ABSTRACT: The water column of the Gotland Deep, a basin with anoxic deep water in the central Baltic, was investigated for its denitrifying microflora in comparison with various chemical, hydrographical and microbiological parameters including *in situ* denitrification rates. Abundance of denitrifying bacteria was determined by the MPN method using nutrient broth plus nitrate medium. As gas formation in inverted vials turned out to be a rather variable feature, N_2O production was used to detect the presence of denitrifying bacteria. Using 4 different cultivation approaches, 77 N_2O producing strains were isolated from the whole water column. Isolates were analyzed for their denitrifying capacity to form NO_2^-, N_2O, and N_2. Taxonomic identification of the strains was done by high resolution electrophoresis of their low-molecular-weight (LMW) RNA (5S rRNA and tRNA). The bulk of the isolated N_2O producing strains (77 %) was identified by their LMW RNA pattern as *Shewanella putrefaciens*. All strains of this species produced N_2O, and NO_2^- and about one third showed N_2-formation. *S. putrefaciens* is considered the most abundant culturable denitrifier of the low oxygen water and the oxic-anoxic interface of the Gotland Deep.

INTRODUCTION

Denitrification plays a major role in the nitrogen budget of the Baltic Sea. The Baltic Sea is the world's largest brackish water environment and is strongly influenced by anthropogenic activity concerning loads of nitrogen and phosphorus (Elmgren 1989). Denitrification is considered a major factor counteracting eutrophication (Rönner 1985). However, little is known about the bacterial microflora catalyzing this part of the nitrogen cycle of the central Baltic (Samuelsson 1985).

The Gotland Deep, the area investigated here, is an anoxic basin, of maximum depth 240 m, in the eastern part of the Baltic Proper, the southernmost and largest part of the Baltic Sea. It is considered representative of the Baltic Proper (Wulff & Rahm 1989). The Baltic Proper is characterized by a halocline at 60 to 90 m depth that inhibits vertical mixing. Below 70 m water exchange relies on horizontal transport processes that are rare and discontinuous events (Stigebrandt & Wulff 1987). With prolonged stagnation periods the deep water displays oxygen deficiency and H_2S accumulates from the sediment. Before our sampling period there had been no major deep water exchange for 9 yr in the Gotland Deep.

Our investigation of the denitrifying microflora was accompanied by a comprehensive study of *in situ* denitrification by means of the acetylene inhibition method (Brettar & Rheinheimer 1991). It was demonstrated in that study that denitrification in the water column of the Gotland Deep is restricted to a narrow layer around the oxic-anoxic interface. These parallel investigations will enable us to compare directly the findings about the denitrifying microflora with *in situ* denitrification rates.

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Denitrifying bacteria have been isolated from many marine environments (Sugahara et al. 1986, see Tiedje 1988 for a review). It is generally assumed that members of the genus *Pseudomonas* are the most abundant denitrifiers in marine as well as freshwater environments (Goering 1985). However, we have to consider that the genus *Pseudomonas* was a very heterogeneous group of bacteria that was divided up in recent years into several new genera based on molecular taxonomy (Palleroni 1984, Woese 1987, Woese et al. 1984). Since all previous studies on denitrifiers and their taxonomic identification were based on classical identification, it is necessary to use the now available molecular identification techniques to place marine denitrifiers within the modern framework of bacterial taxonomy. Thus, our study was geared to analysis of the distribution of denitrifying bacteria in the water column of the Gotland Deep and their taxonomic identification by a molecular technique. This goal was approached by several steps.

First, the abundance of denitrifying bacteria in the water column was obtained by a most probable number (MPN) method using a rather traditional medium, nutrient broth plus nitrate. As criterion for detection of denitrifying bacteria, N_2O formation was used, because gas formation in inverted vials was a rather unreliable feature. Since the *in situ* denitrification layer was the focal point of our interest, sampling emphasis was put on the area near the oxic-anoxic interface.

The second approach involved the creation of a taxonomic data base for the denitrifying bacteria occurring in the water, i.e. their phenotypic and genotypic characterization. Therefore, a variety of strains were isolated using 4 different cultivation techniques, of different selectivity for denitrifiers. An unbiased and rapid taxonomic classification technique was needed if an overview of the taxonomy of a large variety of isolated strains was to be achieved. High resolution electrophoresis of low-molecular-weight (LMW) RNA was used for taxonomic characterization of the strains (Höfle 1990a). By this approach strains could be grouped according to their genotypes. With references available from a data bank, genotypes can be identified according to their profiles of LMW RNA to genus or species level. The same strains analyzed for their genotypes were further investigated for their denitrifying abilities. In this way we created a data base for denitrifying genotypes and could relate them to their denitrifying abilities.

The final approach involved the identification of the most abundant denitrifying genotypes in the water column. The criteria used were (1) their occurrence in the highest dilution series of the nutrient broth plus nitrate media, and (2) their contribution to the overall saprophytic bacteria growing on ZoBell agar. This latter approach has been recently developed (Höfle 1988b, 1990b) and is based on the analysis of the whole assemblage of saprophytic bacteria growing on agar plates using LMW RNA profiles. By this procedure an overview of denitrifying genotypes among the overall assemblage of saprophytic bacteria was obtained.

By using different cultivation approaches, 77 N_2O producing bacterial strains were isolated from the water column of the Gotland Deep. The bulk of the strains were considered to be true denitrifiers, i.e. able to reduce nitrate all the way to N_2O or N_2. The strains were grouped by LMW RNA analysis into 13 different genotypes. The major fraction of the N_2O producing strains were identified as *Shewanella putrefaciens*. Occurrence in the highest dilution series and the molecular analysis of the overall saprophytic assemblage both indicated that *S. putrefaciens* was the most abundant culturable denitrifier, especially in the low oxygen water and the oxic-anoxic interface. Pseudomonads, by contrast were of very minor importance. The possible contribution of *S. putrefaciens* to biogeochemical cycling at the oxic-anoxic interface will be discussed.

MATERIALS AND METHODS

Sampling and field measurements. Samples were taken aboard RV 'Poseidon' from 15 to 17 August 1986 in the Gotland Deep (Stn = BY 15) (57° 20.0' N, 20° 03.0' E) (Brettar & Rheinheimer 1991). Water was collected in 5 and 10 l Niskin PVC-bottles for all chemical parameters. For microbiological parameters samples were taken axenically using sterile champagne bottles mounted on modified ZoBell samplers. In all further handling, care was taken to avoid contamination. Salinity and temperature were determined by a CTD-probe. Oxygen was measured by the Winkler method as described by Grasshoff (1983). Ammonium was determined by the indophenol blue method as modified by Koroleff (1983). Nitrate was reduced by a copper cadmium column to nitrite and determined as nitrite as outlined by Grasshoff (1983). H_2S was determined photometrically by the methylene blue method as modified by Fonselius (1983). The above- mentioned methods are all specified in detail in Grasshoff et al. (1983). These parameters were analysed by the scientific crew of the ship directly after sampling. N_2O was measured by gas chromatography as described by Brettar & Rheinheimer (1991).

Microbiological parameters. Total counts of bacteria were determined by epifluorescence microscopy after staining with acridine orange according to Zimmermann et al. (1978). Samples were fixed in 2 % formaldehyde.

Numbers of saprophytic and denitrifying bacteria were counted after incubation of water samples by 4 different cultivation procedures. As solid medium to count saprophytic bacteria the yeast extract-peptone agar medium (ZoBell-agar, composed of Bacto-peptone (Difco) 5.0 g, yeast-extract 1.0 g, Bacto-agar (Difco) 15.0 g, aged seawater 250 ml, deionized water 750 ml, pH 7.2 ± 0.05; Oppenheimer & ZoBell 1952) was used. On ZoBell agar bacteria were cultivated both aerobically and anaerobically. As liquid media for counting denitrifying bacteria, nutrient broth plus nitrate media were used, at 2 different concentrations of nutrient broth (see below). All media were prepared with natural aged seawater, diluted to a final salinity of 8 ‰. The number of culturable bacteria was estimated by counting colony forming units (CFU) for the solid agar medium and by the MPN-method (de Man 1975) for the liquid media.

Cells growing on yeast extract-peptone agar medium (ZoBell agar) are referred to in the following as saprophytes, aerobic or anaerobic with respect to incubation conditions. ZoBell medium was used as a general microbiological background parameter allowing comparison with many other marine studies (Rheinheimer et al. 1989). ZoBell agar plates were inoculated with 0.1 or 0.01 ml water sample for aerobic counts and with 0.2 ml for anaerobic counts. Anaerobic saprophytes were incubated in a desiccator where oxygen was removed by using the Gas Pak system (Becton Dickinson). CFU were counted after incubation for 14 d in the dark at 20 °C.

Abundance of denitrifying bacteria. For detection of denitrifying bacteria, a nutrient broth plus nitrate medium was used, modified from Sreenivasan & Venkataraman (1956) with the following composition: meat extract (Merck) 3.0 g, Bacto-peptone (Difco) 5.0 g, KNO_3 2.0 g, aged seawater 250 ml, deionized water 750 ml, pH 7.35 ± 0.05. The diluted medium used had the same composition but with a reduced meat extract (0.3 g) and Bacto-peptone (0.5 g) content. Both media were filled into Hungate tubes, and inverted vials (Durham tubes) were added to observe gas formation. The inoculum ranged from 0.001 to 10 ml of the original water sample, applied in 3 replicates. Inoculated cultures were incubated for 5 wk in the dark at 20 °C.

Media with different nutrient broth concentrations were used to provide different concentrations of organic matter to the bacteria. The diluted medium was only used for the layer of our main interest, i.e. around the oxic-anoxic interface.

As a criterion for denitrifying activity in nutrient broth plus nitrate medium, gas formation in the Durham tubes was observed and N_2O in the headspace was measured. To discriminate between gas formation due to N_2 production and due to CO_2 production, the CO_2 concentration was measured additionally. Both N_2O and CO_2 were measured by gas chromatography in the same analytical step.

Gas chromatographic analysis of headspace in Hungate tubes. This analysis for N_2O and CO_2 was applied to all Hungate tubes with nutrient broth plus nitrate medium or thiosulfate nitrate medium, and was used for counting denitrifying bacteria in the water column and/or investigation of bacterial isolates.

Gas samples were taken in the headspace of the Hungate tubes after shaking and equilibration of the liquid medium with the gas phase. To obtain reproducible results for this analysis the volume of liquid medium (14.0 ml) and the headspace (2.7 ml) were of constant size for all samples. Furthermore, a constant temperature of 21 °C was used to ensure the same solubility of N_2O in the medium. After equilibration of the sample at 21 °C, 1 ml of the headspace gas was transferred via a lockable syringe directly to a gas chromatograph.

N_2O and CO_2 concentrations were quantified on a gas chromatograph (model 438A, Packard Instr. Co., Rockville, MD, USA) with an electron capture detector (10 mCi ^{63}Ni) operated at 300 °C. Separation was done on a stainless steel Porapak Q (80/100 mesh) column (3 m, 1/8") at 60 °C and a carrier gas flow of 18 ml min^{-1}. The carrier gas was an Ar/CH_4 (95:5, vol/vol) mixture. Under the running conditions used there was no interference between the measurements of CO_2 and N_2O. The N_2O determinations had a standard deviation of better than 5 % and a detection limit of 500 ppb(v). The CO_2 measurements had a standard deviation of better than 12 % and a detection limit of 1 % CO_2. The measurements were calibrated against standard N_2O and CO_2 mixtures provided by Messer-Griesheim (Duisburg) and Alltech Europe (Brussels). The N_2O and CO_2 concentrations in the samples were calculated according to Weiss & Price (1980).

Gas formation in the Durham tube could be attributed most likely to N_2 production if the amount of N_2O or CO_2 in the head space did not exceed 9.0 %. This amount is calculated based on the assumption that all N_2O and CO_2 formed during the incubation is trapped in the Durham tube. If concentrations higher than 9.0 % occur in the headspace the original concentration in the Durham tube (before shaking and equilibration) may have exceeded the solubility of the gases in the medium trapped in the Durham tube and thus leading to formation of a gas bubble. However, N_2O and CO_2 concentrations rarely exceeded 9.0 %, and gas formation could therefore usually be attributed to N_2 formation. In the few cases where CO_2 or N_2O might be of relevance it will be indicated.

Isolation of bacteria. Bacterial strains were isolated from all media used for counting saprophytic and de-

nitrifying bacteria. From agar plates all different colony types were isolated. From liquid media isolation was done across all dilution series starting with the highest dilution series where growth was observed. All isolation and purification of strains was done by multiple streaking on ZoBell agar. Incubation was done at 20 °C in the dark.

Investigation of denitrifying abilities of isolates. The purified bacterial strains were tested for their denitrifying abilities after inoculation into Hungate tubes with nutrient broth plus nitrate medium (8 g l^{-1} NB). The strains were also inoculated into a thiosulfate nitrate medium according to Baalsrud & Baalsrud (1954) to test for the ability of autotrophic denitrification with thiosulfate as electron donor.

Inoculation of these liquid media was done by transferring strain biomass precultured on ZoBell agar medium. Incubation was done for 5 wk in the dark at 20 °C. Gas formation in Durham tubes, N_2O and CO_2 concentrations was analyzed as described above. NO_2^- was analyzed by the method described in Grasshoff et al. (1983) for cultures with nutrient broth plus nitrate medium. The latter method did not work for the thiosulfate nitrate medium.

Determination of the taxonomic position by LMW RNA analysis. The taxonomic position of the isolates as well as of the bacterial mixtures harvested from the master plates was determined by analysis of their LMW RNA (5S rRNA and tRNA) as described in detail by Höfle (1988a, 1990a). The technique allows grouping of the strains according to genotypes as a result of their distinct LMW RNA pattern after high resolution electrophoresis. Comparison of the patterns for the single genotypes with a reference data bank allows identification of the genotype to species or genus level. In case of lack of references genotypic relatedness among strains within the unknown genus or species usually can still be estimated. Though it is interesting to find the taxonomic identity of a strain, grouping along with the LMW RNA genotypes provides a 'tertium comparationis', i.e. it serves as an unbiased criterion for direct comparison among the strains.

Analysis of mixed cultures on agar plates. The bacterial assemblage growing aerobically on ZoBell agar was analyzed as a whole by LMW RNA analysis, in order to obtain an overview of the abundance of denitrifying bacteria among saprophytic bacteria on ZoBell agar. For this purpose, master plates were generated and the entire biomass harvested from these agar plates was subjected to LMW RNA analysis (Höfle 1988b, 1990b). To generate master plates, 50 colonies were randomly picked from the original agar plates used for saprophytic counts. After incubation for 2 wk at 20 °C in the dark the whole biomass was harvested and analyzed. Master plates were preferred over the original plates as they produce a more uniform biomass per colony. This was achieved by transferring a rather large and uniform amount of each original col-

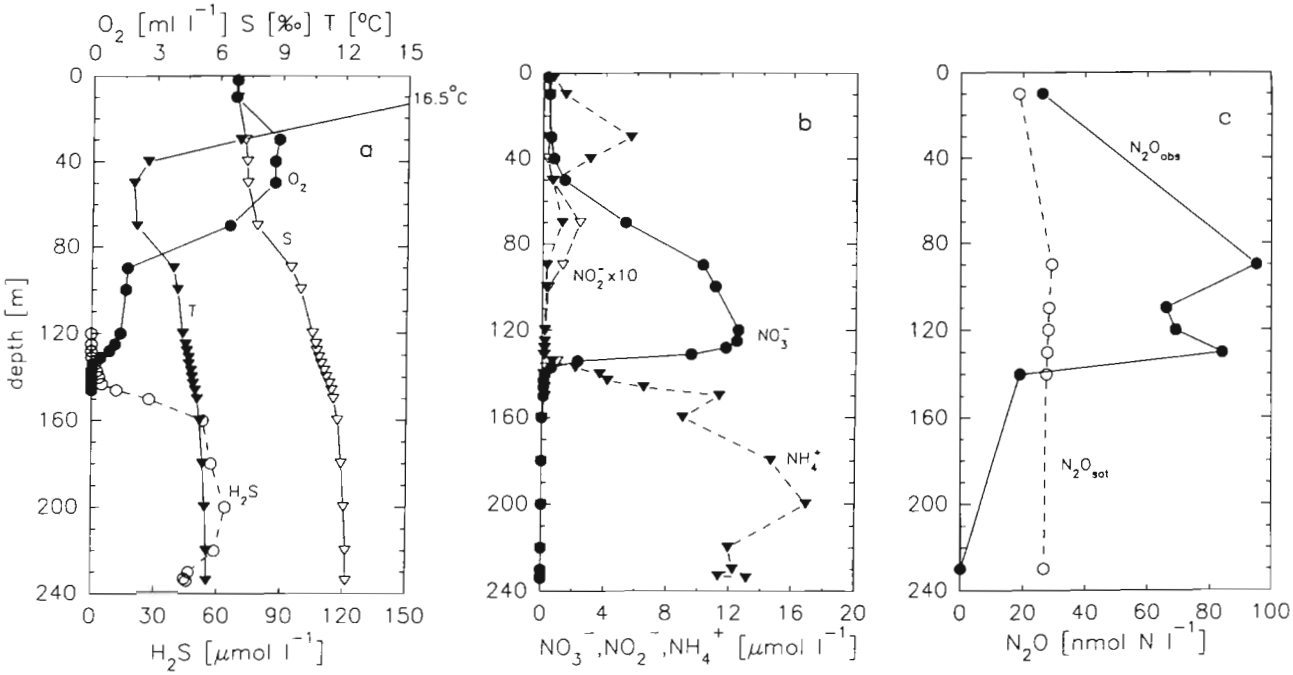

Fig. 1. Depth profiles of the hydrographical and chemical parameters in the water column of the Gotland Deep in summer 1986. (a) Temperature, salinity, oxygen and hydrogen sulfide; (b) vertical distribution of NO_3^-, NO_2^- and NH_4^+; (c) observed N_2O concentrations (N_2O_{obs}) and calculated saturation values (N_2O_{sat})

ony with toothpicks to the master plate. The templates for production of the master plates were identical with the plates from which single colonies had been isolated in order to test their denitrifying abilities and analyze their genotypes. This enabled us to correlate the genotypic composition of the bacterial assemblage on the agar plates to its physiological properties. More details on the analysis of mixed cultures using their LMW RNA are given elsewhere (Höfle 1990b).

RESULTS

In situ measurements

Depth profiles of physical, chemical and microbiological parameters in the water column of the Gotland Deep are summarized in Fig. 1. These profiles, obtained in 1986, are fairly typical for the summer situation during periods of stagnation of deep water (Rheinheimer et al. 1989, Brettar & Rheinheimer 1991, 1992). The reproducibility of the situation is evident when compared with an investigation in summer 1987 (Brettar 1991).

The water column was characterized by 2 density gradients: the thermocline at 20 m and the halocline at 60 to 90 m depth. The thermocline formed the lower boundary of the layer of phytoplankton primary production (Gocke 1989). Oxygen was close to saturation level (> 80 %) in the water column above the halocline, and decreased strongly within the halocline. The oxic-anoxic interface was at around 130 m depth. Below the interface, H_2S concentrations increased with depth (Fig. 1a).

In general, nitrate concentrations were low above the halocline. Within and below the halocline nitrate increased up to 11 µmol l^{-1}. Typically a pronounced peak was observed above the oxic-anoxic interface followed by a strong decline within the interface layer. At the top of the H_2S layer very low NO_3^- concentrations occurred in the presence of low concentrations of H_2S. Nitrate disappeared completely with increasing H_2S concentrations (> 10 µmol l^{-1}) in the deeper part of the anoxic layer. Ammonium concentrations were usually very low in the oxic part of the water column. High concentrations occurred in the anoxic part of the water column, where NH_4^+ showed a similar increase to H_2S (Fig. 1b).

Nitrous oxide concentrations were close to the at-

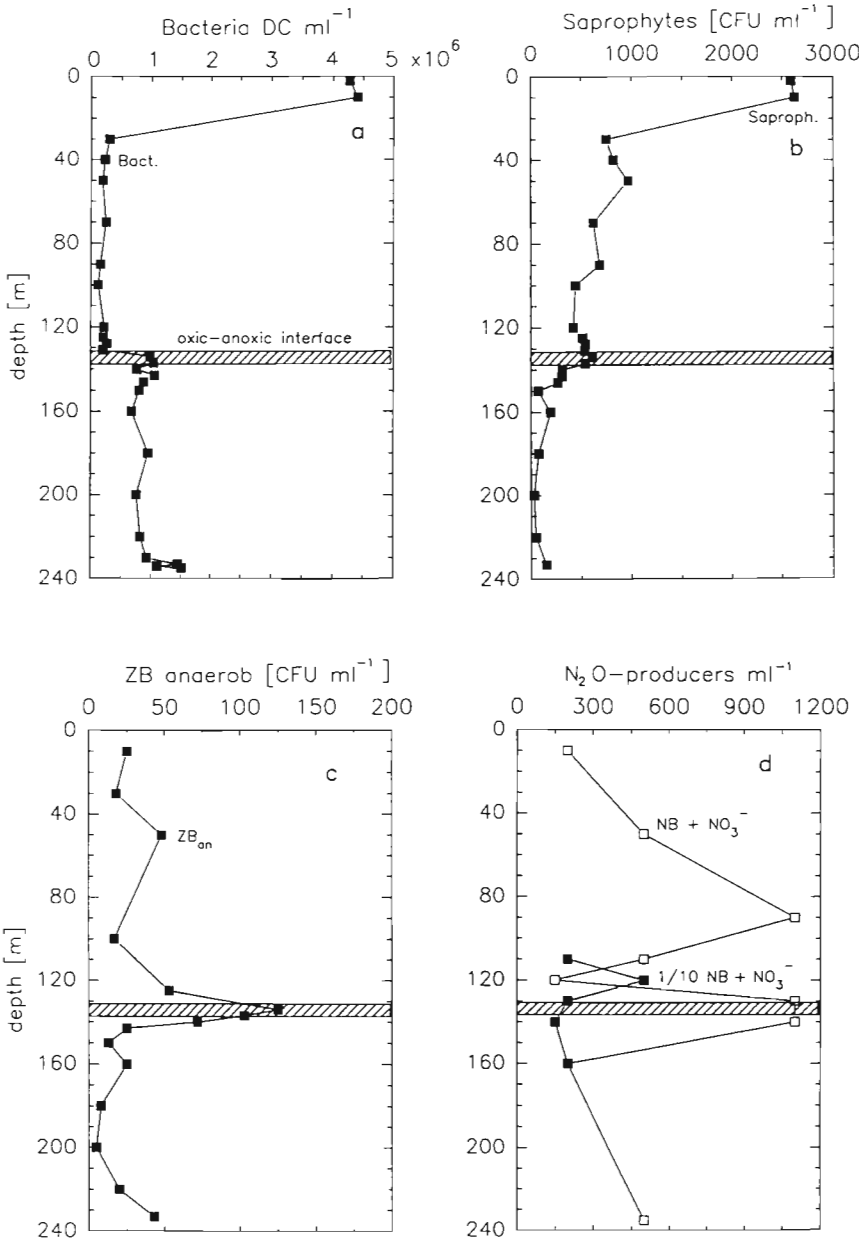

Fig. 2. Depth profiles of microbiological parameters in the water column of the Gotland Deep. (a) Bacterial total numbers (direct counts (DC) by epifluorescence microscopy); (b, c) counts of saprophytic bacteria growing aerobically (b) and anaerobically (c) on ZoBell agar; (d) numbers of bacteria producing N_2O in nutrient broth plus nitrate medium (NB + NO_3^-) and 1:10 diluted nutrient broth plus nitrate medium (1/10 NB + NO_3^-)

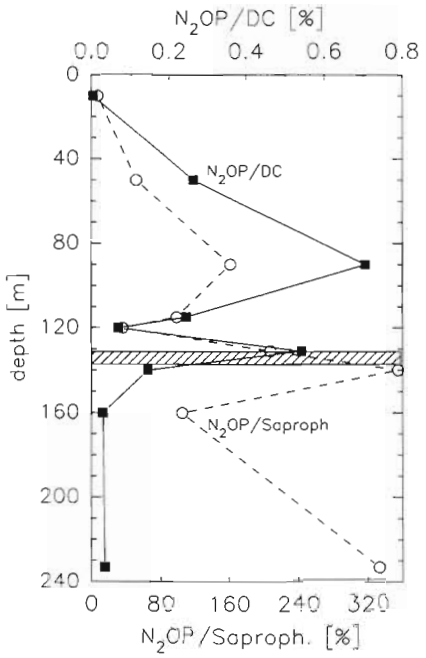

Fig. 3. Percentage of N_2O producing bacteria (medium: NB + NO_3^-) compared to bacterial direct counts by epifluorescence (N_2OP/DC) and to saprophytic (aerobically grown) bacteria ($N_2OP/Saproph.$)

mospheric saturation value in the water column above the halocline. Supersaturation was always found in the water of the lower part of the halocline and below the halocline. The maximum value of N_2O saturation was 330 % at 90 m depth. Nitrous oxide was undersaturated in the oxic-anoxic interface and depleted in the anoxic deep water (Fig. 1c).

All microbiological profiles are given in Fig. 2. Total bacterial numbers (direct counts by epifluorescence microscopy) were in the range 0.1×10^6 to 4.4×10^6 cells ml^{-1}. The maximum occurred in the euphotic zone. Less pronounced peaks were in the near-sediment water layer (about 1.5×10^6) and at the oxic-anoxic interface (about 1.0×10^6 cells). Total bacterial numbers were always lowest in the oxic water between the euphotic zone and the oxic-anoxic interface, ranging from 0.1 to 0.3×10^6 cells ml^{-1} (Fig. 2a).

The numbers of saprophytes growing aerobically on ZoBell medium ranged between 30 and 2600 CFU ml^{-1} with highest values in the euphotic zone and lowest values in the anoxic water. Anaerobically grown saprophytes displayed much lower values with a range from 5 to 170 CFU ml^{-1}. Maximum counts were recorded in the layer of the oxic-anoxic interface. The percentage of aerobic and anaerobic saprophytes compared to total bacterial numbers counted by epifluorescence ranged from 0.4×10^{-2} to 0.5 %, and 0.6×10^{-3} to 0.03 %, respectively (Fig. 2b,c).

The number of bacteria producing N_2O in nutrient broth plus nitrate medium (8 g l^{-1} NB) ranged from 150 to 1100 bacteria ml^{-1}. Maxima occurred at the lower boundary of the halocline (90 m) and at the oxic-anoxic interface (130 to 140 m). This corresponded to 0.5×10^{-2} to 0.7 % of the total bacterial numbers counted for the same samples (Fig. 2d).

Fig. 3 shows the percentage of N_2O producing bacteria compared to total bacterial counts and aerobic saprophytes in the water column. The highest percentage of N_2O producing versus total counts occurred at the bottom of the halocline (90 m, 0.70 %) and at the oxic-anoxic interface (130 m, 0.54 %). Compared to the aerobic saprophytic bacteria growing on ZoBell agar the numbers of N_2O producing bacteria were in the same order of magnitude. Values of saprophytic bacteria exceeded the N_2O producing bacteria in the euphotic layer, while N_2O producing bacteria exceeded the number of saprophytes by a factor of 3 at the interface and in the near-sediment (235 m) layer.

The number of bacteria producing N_2O in 1/10 diluted nutrient broth plus nitrate medium (0.8 g l^{-1} NB) was only recorded for the layer around the oxic-anoxic interface from 110 to 160 m depth. Their number ranged from 150 to 500 bacteria ml^{-1} (Fig. 2d). In both nutrient broth plus nitrate media gas formation was a rather sporadic event, e.g. gas formation occurred in one dilution step twice but not in any of the tubes with a larger inoculum. It was therefore impossible to evaluate gas formation by means of the MPN-tables (de Man 1975), and N_2O-formation is used instead as a criterion for counting denitrifiers.

N_2O producing bacterial isolates

Out of 123 strains isolated from the water column of the Gotland Deep, 77 were able to produce N_2O after inoculation into nutrient broth plus nitrate medium. The N_2O producing strains were investigated further for CO_2, NO_2^- and gas production. In thiosulfate nitrate medium, none of the 123 strains showed visible growth, or was able to produce N_2O. For monitoring autotrophic CO_2 uptake the gas chromatographic CO_2 detection method was not sensitive enough; CO_2 production in the thiosulfate medium was never observed.

Results of the quantitative measurements for N_2O, CO_2, and NO_2^- and the qualitative observation of gas formation in the Durham tubes of cultures in nutrient broth plus nitrate medium are listed in Table 1. The results are sorted for the different genotypes (see below). N_2O concentrations in the head space ranged from 288 ppm(v) to 24 % (= 240 000 ppm(v)) for the total of all strains. This corresponds to a range for the amount of nitrate reduced to N_2O from 0.02 to 16.0 mmol l^{-1} NO_3^-. At an initial nitrate concentration in the medium

Table 1. N_2O producing bacterial isolates from the water column of the Gotland Deep. Amounts of N_2O, NO_2^- and CO_2 produced, and gas formation in the standard denitrification assay in nutrient broth plus nitrate medium are given for the different genotypes (GT = genotype signature, N = number of strains per genotype): mean values ($\bar{x}$), standard deviations (SD) and ranges are given for N_2O, CO_2 and NO_2^- production; for gas formation the percentage of gas producing strains among all strains of a genotype is shown; for signatures of genotypes (GT) see Table 2

Genotype GT	(N)	N_2O (ppm) $\bar{x}$ (± SD) (range)	CO_2 (%) $\bar{x}$ (± SD) (range)	Gas	NO_2^- (mmol l^{-1}) (N1)[a]	$\bar{x}$ (± SD) (range)	Depth (m)
S	(59)	1161 (±481) (312–2799)	4.3 (±1.4) (1.4–8.4)	31 %	(26)	2.1 (±0.6) (1.3–3.0)	50, 90, 110, 120, 130, 140, 160, 180, 235
E2	(2)	1383 (±368) (1122–1643)	6.7 (±0.0) (6.7–6.7)	100 %	(2)	4.0 (±0.4) (3.8–4.3)	120
E3	(2)	405 (±110) (327–483)	4.5 (±4.5) (1.4–7.7)	50 %		–	10, 130
E4	(3)	2667 (±1185) (1317–3529)	10.7 (±3.7) (6.5–13.4)	100 %[b]	(2)	3.6 (±0.5) (3.3–4.9)	10, 130, 140
E5	(1)	601 – –	2.2 – –	0 %	(1)	4.5 – –	110
P1	(1)	3413 – –	3.6 – –	100 %	(1)	7.9 – –	110
P2	(1)	288 – –	2.3 – –	100 %		–	10
V3	(1)	725 – –	3.0 – –	0 %	(1)	1.9 – –	120
C	(1)	670 – –	2.3 – –	0 %		–	10
D	(3)	235 660 (±11 266) (22 194–242 490)	2.4 (±0.6) (1.9–3.0)	100 %[b]	(3)	0.0 (±0.0) (0.0–0.0)	120, 130
H	(1)	802 – –	4.0 – –	0 %	(1)	1.2 – –	235
K	(1)	8685 – –	1.9 – –	0 %		–	130
R1	(1)	633 – –	3.0 – –	100 %		–	235
Total: 13	(77)			39 %			

[a] Number of strains per genotype tested for NO_2^- production. All strains isolated were tested for N_2O, CO_2 and gas production
[b] Gas formation of the strains could be due to N_2O or CO_2 production

of 20 mmol l^{-1}, up to 80 % of the nitrate was recovered as N_2O.

Gas formation, i.e. formation of gas bubbles in the Durham tubes, was observed only for strains that produced N_2O. Of the N_2O producing strains, 39 % showed gas formation. NO_2^- concentrations in the medium ranged from 0 to 7.9 mmol l^{-1}. CO_2 concentrations in the head space ranged from 1.4 to 13.4 %, corresponding to a production of 0.6 to 6.0 mmol l^{-1} CO_2 in the medium.

The 77 N_2O producing strains were grouped taxonomically using low-molecular-weight (LMW) RNA analysis. This grouping resulted in 13 different genotypes of N_2O producing bacteria. Of the N_2O producing bacteria, 77 % were identified as *Shewanella putrefaciens*. The remaining 18 strains were rather diverse. They could be grouped into 12 different genotypes, which could be partially identified to genus or species level (Table 2) (Höfle 1990b). Seven strains i.e. 9 % of the N_2O producing strains, could not be identified.

All strains isolated were characterized by a discrete range concerning their amount of N_2O, CO_2 and NO_2^- production. Standard deviation for duplicate cultures of the single strains in the Hungate tubes ranged on average for N_2O between 1 and 10 % of the mean, for

Table 2. List of N_2O producing genotypes isolated from the water column of the Gotland Deep: Identification according to LMW RNA analysis of genotype

Genotype signature	Genus	Species
Genotypes identified to species or genus level:		
S	*Shewanella*	*putrefaciens*
E2	Enterobacteriaceae[a]	
E3	Enterobacteriaceae[a]	
E4	Enterobacteriaceae[a]	
E5	Enterobacteriaceae[a]	
P1	*Pseudomonas*	sp.
P2	*Pseudomonas*	*aeruginosa*
V3	*Vibrio*	*anguillarum*
Unidentified genotypes: C, D, H, K, R1		

[a] Similar to *Enterobacter aerogenes* and *Escherichia coli* (see Höfle 1990b)

CO_2 between 3 and 35 %, and for NO_2^- between 11 and 20 %. Gas formation was a rather variable feature. About half (53 %) of the gas forming strains showed gas formation in only 1 tube of the 2 replicates. By contrast, concentrations of N_2O, CO_2 and NO_2^- were similar in these replicate cultures.

The genotypic groups exhibited a larger standard deviation than replicate cultures of the single strains but still showed a discrete pattern for their amount of N_2O, CO_2 and NO_2^- production. For N_2O production the standard deviation expressed in percentage of the mean ranged between 5 and 45 %, for CO_2 between 0 and 100 %, and for NO_2^- between 10 and 30 % (see Table 1).

Shewanella putrefaciens was isolated by all different cultivation approaches used from samples of 50 m down to 235 m. Strains identified as *S. putrefaciens* displayed on the average 1160 ppm N_2O and 4.3 % CO_2 in the headspace of the Hungate tube and 2.1 mmol NO_2^- in the medium. Standard deviation for N_2O for all strains was 41 % for N_2O, 33 % for CO_2 and 27 % for NO_2^-. Of the *S. putrefaciens* strains, 31 % showed gas formation. Gas formation was a rather variable feature with only 3.4 % of the gas forming strains displaying gas formation in both replicates.

Abundance of N_2O producing bacterial genotypes in the water column

Two criteria were used for detection of the most abundant N_2O producing bacterial genotype of the water column: (1) occurrence in the highest dilutions of the liquid media (diluted and undiluted nutrient broth plus nitrate) used for counting N_2O producing bacteria and (2) the abundance on aerobically incubated ZoBell agar plates used for counting saprophytic bacteria. The results for the dilution series are derived from direct analysis of the respective strains. Abundance of N_2O producing genotypes among saprophytic bacteria comes from overall LMW RNA analysis of the bacterial biomass of the respective master plates. Table 3 displays the N_2O producing genotypes isolated from the highest dilution steps showing N_2O production, i.e. tubes inoculated with 0.01 or 0.001 ml original seawater sample. For the undiluted nutrient broth medium the whole water column was studied, for the 1/10 diluted medium only the layer around the oxic-anoxic interface was investigated. The table displays furthermore the percentage of the biomass of N_2O producing genotypes compared to the total bacterial biomass growing on the ZoBell agar master plates.

In nutrient broth plus nitrate medium, *Shewanella putrefaciens*, 2 Enterobacteriaceae (E4, E5) and an unidentified genotype occurred in the highest dilution steps. In the layer close to the interface (120 m, 130 m), *S. putrefaciens* was most abundant. In the diluted nutrient broth plus nitrate medium, *S. putrefaciens* was the only N_2O producing isolate obtained for the investigated layer between 110 and 160 m. On the ZoBell

Table 3. Abundance of N_2O producing bacterial genotypes according to their occurrence in different media. The media were inoculated with original seawater samples and were used concomittantly for estimation of the number of denitrifying ($NB+NO_3^-$ and 1/10 $NB+NO_3^-$ media) and saprophytic bacteria (ZoBell agar) as well as for isolation and identification of bacteria. For signatures of genotypes see Table 2. (a,b) N_2O producing genotypes in highest dilutions of $NB+NO_3^-$ and 1/10 $NB+NO_3^-$ media. (c) N_2O producing genotypes in percentage of the biomass of the total assemblage growing aerobically on ZoBell agar (for details see Höfle 1990 b)

Depth (m)	(a) $NB+NO_3^-$ 10^{-2}	10^{-3}	(b) 1/10 $NB+NO_3^-$ 10^{-2}	10^{-3}	(c) ZoBell agar (% of total assemblage)
10	E4	o[a]	–[b]		0 %
30	–		–		0 %
50	x[c]	x	–		0 %
90	–		–		32 % S
110	E5	x	S	o	–
120	S	x	S	o	64 % S
130	o	S	o	S	48 % S
140	–		S	x	80 % S
160	x	x	S	o	–
180	–		–		0 %
235	o	R1	–		58 % S

[a] o = no isolate obtained
[b] – = not determined
[c] x = isolate unable to produce N_2O

agar master plates, *S. putrefaciens* was the only N_2O producing isolate that could be detected by this overall analytical method. Between 90 m and 235 m, 32 to 80 % of the total bacterial biomass growing on the agar plates could be accounted for by *S. putrefaciens*. According to the observations on all 3 media, *S. putrefaciens* was the most abundant culturable denitrifying species in the low oxygen and anoxic water of the Gotland Deep with a most pronounced abundance in the layer around the oxic-anoxic interface.

DISCUSSION

Nitrous oxide production as indicator for denitrifying bacteria

Nitrous oxide production was demonstrated to be a very reliable and reproducible feature of the bacterial isolates. This could be shown most clearly by the investigation of the 77 N_2O producing strains. Duplicate incubations of the same strains showed for the amount of N_2O produced an average standard deviation of the mean of 1 to 10 %. In no case did a strain known as N_2O producer fail to produce N_2O in nutrient broth plus nitrate medium.

By contrast, gas formation i.e. formation of gas bubbles in Durham tubes was a highly variable feature. Of the nitrous oxide producing strains, 39 % produced gas, of which more than half (53 %) of the gas producing strains produced gas only in one of the duplicate cultures. Gas formation was observed for 8 of the 13 N_2O producing genotypes, with 6 out of 8 showing variable gas formation.

Since CO_2 and N_2O could be excluded as a source for gas formation, gas formation could be attributed most likely to N_2 formation. Therefore, we can assume that all gas forming strains, i.e. 39 % of the N_2O producing strains are able to denitrify NO_3^- to N_2. Taking into account that gas formation is rather easy to disturb, it can be assumed that 39 % is the lowest estimate of strains able to denitrify to N_2. It is likely that the true number of strains able to denitrify to N_2 greatly exceeds 39 %. For example, assuming that not just 31 % but all *Shewanella putrefaciens* strains are able to denitrify, would increase the fraction of N_2 producing bacteria to 92 % of all isolated N_2O producing bacteria.

Taking into account the variability of gas formation, it is obvious that denitrifiers can be easily missed by using gas formation as a criterion. Patriquin & Knowles (1974) therefore recommended using N_2O production as a criterion for denitrification instead of gas formation.

According to our experience with the isolated strains we consider N_2O production in nutrient broth plus nitrate medium a reliable criterion for counting denitrifying bacteria, as most of the genotypes producing N_2O were likely to be true denitrifiers on the one hand, and showed a variable gas formation concomittant with a rather stable N_2O production on the other. It is possible that one might overestimate the true numbers of denitrifiers in this way, but we consider this potential source of error small compared to the severe underestimation that is very likely to occur by using gas formation as the only criterion for denitrification.

Denitrifying bacteria as significant fraction of the heterotrophic microflora in low oxygen and anoxic water

The number of N_2O producing bacteria will be used here as an estimate of the number of the denitrifying bacteria. The number of bacteria able to produce N_2O in diluted and undiluted nutrient broth plus nitrate medium was in the same order of magnitude as the number of aerobic saprophytes growing on ZoBell agar. Also, the ratios of N_2O producing bacteria and saprophytes to total bacterial epifluorescent counts were rather similar. The major difference is the distribution pattern in the water column. Aerobic saprophytes had their maximum in the euphotic layer (10 m). At the oxic-anoxic interface there was only a minor increase. N_2O producing bacteria displayed very low values in the euphotic layer and had maxima at the bottom of the halocline and at the oxic-anoxic interface (Fig. 2).

This pattern is also reflected by the fraction of N_2O producing bacteria among the saprophytic bacteria growing aerobically on ZoBell agar (Table 3): N_2O producing bacteria were undetectable in the well-oxygenated water down to 50 m. The fraction of N_2O producing bacteria increased with the onset of low oxygen concentrations at the bottom of the halocline (90 m). Except for the sample from 180 m, N_2O producing bacteria formed a major fraction of the saprophytic bacteria in the low oxygen and anaerobic water.

The availability of oxygen may be a regulating factor for the increasing occurrence of denitrifying bacteria in the water column and a reason for their higher contribution to the overall saprophytic bacteria. Another important factor for the abundance of denitrifying bacteria may be the availability of organic carbon. Tiedje (1988) demonstrated the relevance of carbon for the abundance of denitrifiers in many ecosystems including aquatic ones. Both factors, oxygen and carbon, may have led to the pattern of denitrifiers we obtained in the water column of the Gotland Deep. In low oxygen water, maxima can be expected when carbon availability is high. This is most likely at (1) the bottom of the halocline, (2) in the layer around the oxic anoxic interface, and (3) in the vicinity of the sediment. A

higher availability of organic carbon at the bottom of the halocline may result from the retardation of the sinking speed to which sinking particles are subjected in the layer of the halocline due to the density gradient. The oxic-anoxic interface is a layer of high chemoautotrophic production due to oxidation of reduced sulfur compounds (Gocke 1989, Brettar & Rheinheimer 1991). Grazing and leakage of organic nutrients may result here in an enhanced availability of organic carbon as indicated by the increase of amino acid concentrations at the interface (Mopper & Lindroth 1982). The sediment-near water may display higher concentrations due to input of sedimented material.

Shewanella putrefaciens as most abundant denitrifier in the low oxygen and anoxic water column

Shewanella putrefaciens turned out to be the most abundant N_2O producing bacterium in the low oxygen and anoxic water of the Gotland Deep. This could be shown by the dilution series with nutrient broth nitrate medium as well as by analyzing the overall bacterial community growing on ZoBell agar. The highest abundance in terms of absolute numbers as well as fraction of the overall saprophytes was observed in the layer around the oxic-anoxic interface.

The abundance of *Shewanella putrefaciens* only refers to the culturable fraction of the heterotrophic bacterial community. Among this fraction *S. putrefaciens* can be considered to play a dominant role in the low oxygen and anoxic water. Compared to the total bacterial numbers counted by epifluorescence, the culturable bacterial fraction never exceeded 1 %, and tended to range between 0.1 and 0.01 %.

Shewanella putrefaciens can be considered as a true denitrifier due to gas production that could most likely be attributed to N_2 production. We assume that most of the strains were able to produce N_2 because all measured features except gas formation showed a rather high homogeneity. This makes it likely that also N_2 production is a homogeneous feature of the *S. putrefaciens* strains. N_2 production may have been easily disturbable and therefore often missed by using only 2 replicates for cultivation of the strains. We therefore assume that the bulk of the *S. putrefaciens* strains can be regarded as denitrifying bacteria able to reduce NO_3^- all the way to N_2.

Possible relevance of *Shewanella putrefaciens* for the biogeochemical cycling in the water column of the Gotland Deep

The high abundance of *Shewanella putrefaciens* in the low oxygen and anoxic water of the Gotland Deep and the pronounced presence at the oxic-anoxic interface makes it likely that *S. putrefaciens* plays a significant role for the turnover of organic matter, especially at the oxic-anoxic interface.

As the strains of *Shewanella putrefaciens* isolated from the interface were able to denitrify as well as grow aerobically, a potential role for turnover of organic matter using NO_3^- or oxygen as electron acceptor can be considered.

However, our investigations about denitrification in the Gotland Deep performed at the same time as the isolation of the bacteria showed that denitrification can be considered to be mostly a chemoautotrophic process (Brettar & Rheinheimer 1991). Denitrification as measured by the acetylene inhibition method was restricted to a narrow layer of the oxic-anoxic interface with concomittant occurrence of H_2S and NO_3^-. Reduced sulfur compounds provided by the anoxic water obviously were used as electron donor for driving a chemolithotrophic denitrification. A high chemolithotrophic potential for this layer was demonstrated by Gocke (1989).

The role of *Shewanella putrefaciens* may be explained by 2 different scenarios. First, the high chemolithoautotrophic activity at the oxic-anoxic interface could be a source for a large amount of organic carbon that partially could have been accessible to the heterotrophic *S. putrefaciens*. In particular grazing by flagellates may have caused transfer of organic carbon from the autotrophic microorganisms to *S. putrefaciens* by release of dissolved organic carbon via excretion or sloppy feeding. A large number of flagellates were indeed observed at the interface (H. Kuosa, H. Galvão, pers. comms.).

The second scenario attributes a major role for the turnover of organic carbon to *Shewanella putrefaciens* after sedimentation events of algal blooms. Especially after sedimentation of the spring bloom, the water column is enriched with organic carbon (Stigebrandt & Wulff 1987). In this case the denitrification layer may be extended upwards into the low oxygen layer as shown by Rönner & Sörensson (1985). In this case a low oxygen and anoxic layer may occur above the H_2S containing water. The availability of carbon may enable heterotrophic denitrification above the H_2S containing water, which could now be performed by *S. putrefaciens*. The presence of *S. putrefaciens* in this low oxygen layer could be interpreted as a part of a population left over from the sedimentation of the spring bloom. In this case *S. putrefaciens* could be regarded as an r-strategist waiting for the arrival of a larger amount of organic carbon. The very rapid growth of *S. putrefaciens* on ZoBell agar even after prolonged periods of starvation (e.g. after storage in sea water for years, *S. putrefaciens* recovers overnight) is consistent with such a role.

The potential for denitrification by *Shewanella putrefaciens* strains was also demonstrated by Nealson et al. (1991) and Samuelsson (1985). The *S. putrefaciens* strain that was investigated by the latter author originated from the deep water of the Baltic proper. In addition to denitrifying abilities this strain also showed the capability of dissimilative reduction of nitrate to ammonium.

Another important aspect for the biogeochemical relevance of *Shewanella putrefaciens* is the potential to use a large array of different electron acceptors. According to Myers & Nealson (1988) *S. putrefaciens* is able to utilize, besides oxygen and nitrate, 8 other electron acceptors. The potential to use iron, manganese and sulfite may be especially advantageous in layers of transition from oxic to anoxic conditions.

Assuming a similar versatility concerning the use of electron acceptors for the *Shewanella putrefaciens* strains of the Gotland Deep, *S. putrefaciens* could play a role in the oxidation of organic carbon both in the scenario of a narrow denitrification layer at the oxic-anoxic layer as well as in the scenario of an extended denitrification layer in a low oxygen or anoxic layer above the H_2S containing water. In both cases a variety of electron acceptors could be used for consumption of available and utilizable organic carbon. Assuming a higher availability of carbon in the case of an extended anoxic layer above the H_2S containing water, a prominent role could be attributed to *S. putrefaciens*. In this latter case *S. putrefaciens* would be able to use the organic carbon by reducing all electron acceptors available. Nealson et al. (1991) showed the effect of such a stepwise use of oxygen, nitrate, manganese and iron reduction after sedimentation of a bloom in the Black Sea. Their investigations indicated that *S. putrefaciens* is likely to have played a major role in the reduction of manganese and iron. The occurrence of a similar situation of stepwise reduction of different electron acceptors after the spring bloom can be deduced from observations of Dyrssen & Kremling (1990) in the Gotland Deep in late spring 1981. Their data on dissolved iron and manganese in the water column may hint on a similar role of these elements as electron acceptors under conditions of high carbon supply as was reported from the Black Sea (Nealson et al. 1991).

It thus appears likely that *Shewanella putrefaciens* could play a major role in the biogeochemical cycles in the Gotland Deep especially for heterotrophic processes in the low oxygen water and the transition from oxic to anoxic conditions. Oxygen as well as nitrate may have been used as electron acceptors. The use of manganese and iron is likely to play a major role after sedimentation events that increase the availability of organic carbon and thus promote the consumption of oxygen and nitrate at the oxic-anoxic interface. In such a situation of an anoxic layer above the H_2S layer the advantage of a highly versatile microorganism able to use all available electron acceptors at a given high carbon supply is most obvious. Whether or not this versatility and the corresponding advantage could also occur for the *S. putrefaciens* strains at the narrow oxic-anoxic interface as in our case still needs further research.

Conceivable reasons for the unusual spectrum of denitrifying microorganisms of an aquatic ecosystem

As outlined by Goering (1985) and Tiedje (1988) pseudomonads should be expected as dominant denitrifying microorganisms for marine and freshwater ecosystems. In our study, however, true members of the genus *Pseudomonas* turned out to be of very minor importance. The only 2 *Pseudomonas* strains able to denitrify had a rather low abundance, i.e. never occurring in higher dilution series of the MPN tubes (P1 occurred in a tube with 10 ml inoculum, P2 was isolated from ZoBell agar).

This discrepancy compared to other studies may have different reasons. First, the conditions in the ecosystem itself, i.e. brackish water and the occurrence of anoxic H_2S containing deep water, may have caused the establishment of a population especially adapted to these conditions. This reason may hold especially for the high abundance of *Shewanella putrefaciens*, a species that is perfectly adapted to oxic-anoxic transition zones. The high abundance of this species in the Black Sea may indicate furthermore the oxic-anoxic transition as a major selection factor for this species.

A marine ecosystem where pseudomonads also did not play a role as denitrifiers were the sediments of the northwest African upwelling region. Rüger (1985) showed that the bulk of the denitrifying microflora belonged to 2 species, later on described as 2 new species of marine agrobacteria (Rüger & Höfle 1992).

The second reason is the use of an unbiased molecular method for identification of the isolates allowing a clear-cut definition of the genotype of the strains with the further possibility to identify the genotype to genus or species level. The advantage of such an unbiased method is most evident by regarding the genus *Pseudomonas* (Höfle 1992a). Before the advent of molecular identification techniques this genus was a rather heterogeneous group that included many non-pseudomonads. Identification according to classical taxonomic criteria using phenotypic tests resulted in many non-pseudomonad strains being assigned to the genus *Pseudomonas*. This was also the case for *Shewanella putrefaciens* which was previously termed *P. putrefaciens* (MacDonell & Colwell 1985). (It should

be mentioned here that *S. putrefaciens* was also called *Alteromonas putrefaciens* after it was excluded from the genus *Pseudomonas*.) In this way a rather distinct group of denitrifiers was hidden in the large pool of pseudomonads.

In cooperation with M. Bölter & G. Rheinheimer a numerical taxonomy method (Bölter 1977) was also applied to our isolates from the Gotland Deep. As was to be expected, most isolated denitrifiers, including the *Shewanella putrefaciens* strains, were classified as *Pseudomonas* spp. (Bölter & Rheinheimer pers. comm.).

Therefore, we think, a combination of genotyping and phenotyping is needed if we want to achieve insight into abundance and function of the bacteria in the ecosystem. For genotyping we suggest molecular methods for an unbiased identification or at least genotyping of the strains. Independent from molecular identification, investigation of physiological features, in our case denitrifying abilities, is needed to improve understanding of the potential function of the bacteria in the ecosystem.

By the dual approach of phenotyping and genotyping it is conceivable that a unifying picture of the ecological functioning of specific bacterial groups may be generated. Via the concomittant knowledge of molecular and physiological characteristics, laboratory findings can be related to *in situ* activities of microorganisms.

During all these considerations we have to keep in mind that the culturable bacteria only represent a minor fraction of the total bacterial community (less than 1 %), with the major unculturable fraction remaining unknown. The advent of molecular techniques will enable further insight into the overall structure of the bacterial communities (Höfle 1992b). However, results obtained by molecular techniques will also need knowledge gained from bacterial strains of the respective ecosystem to relate the observed structure of the bacterial community to its potential activities. For a final interpretation of the biogeochemical potential of bacterial communities in ecosystems, information derived from the investigation of culturable strains remains therefore essential.

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