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Denitrifiers in Sediments of the Weser Estuary and the German Bight: Densities of Nitrate-Dissimilating and Nitrite-Dissimilating Bacteria

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Abstract: At 24 stations in the Weser Estuary and the German Bight the Most Probable Numbers (MPN/g dry wt. sediment) of nitrate-dissimilating (= denitrifying) and of nitrate plus nitrite-dissimilating bacteria were recorded. The numbers of nitrite-dissimilating bacteria, i. e. denitrifiers not capable of reducing nitrate to nitrite, were calculated by subtraction of the MPN for nitrate-dissimilating from the MPN of nitrate plus nitrite-dissimilating bacteria. By determining the percentages of these bacteria in relation to the number of the heterotrophs, the ecological importance of denitrification, especially the nitrite dissimilation, was estimated. The results showed the MPN of nitrate-dissimilating bacteria to be in the range of 0-156 (up to 0.8 % of heterotrophic bacteria). An exception was the sediment of one station with a MPN of 1849, or 5.2 % of the heterotrophs. The amounts of nitrite-dissimilating bacteria were between 0 and 2352 (up to 13 % of heterotrophic bacteria). In the estuary the numbers of nitrate-dissimilating and of nitrite-dissimilating bacteria showed a decreasing tendency with distance from Bremerhaven. The highest numbers were found in the Weser off Bremerhaven and also at 3 stations in the German Bight, south of the Isle of Helgoland.

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Since YUATT (1954) first described the reduction of nitrite but not nitrate to nitrogen gas by an *Achromobacter* strain, a few other bacteria have become known with the same trait. CHATELAIN (1969) found that 11 strains of *Alcaligenes odorans* var. *viridans* were able to reduce nitrite but not nitrate to elementary nitrogen. PICHINOTY et al. (1978) in their recent study on the physiology and taxonomy of *Alcaligenes denitrificans, A. odorans* and *A. faecalis* confirmed the report of CHATELAIN (1969). Three *Pseudomonas* strains isolated from Oregon soils were found to be incapable of nitrate dissimilation, but were capable of reducing nitrite to nitrogen gas (VANGNAI and KLEIN 1974). A denitrifying bacterium of the genus *Flavobacterium* not reducing nitrate but nitrite was also isolated by PICHINOTY et al. (1976). As cited by CHATELAIN (1969) from other publications, numerous strains from the genera *Neisseria* and *Lactobacillus* did not reduce nitrate, but nitrite could be reduced and certain strains of *Pseudomonas aeruginosa* also produced gaseous nitrogen from nitrite but not from nitrate.

Using a nutrient broth with the addition of 0.5 % KNO_3 as an enrichment technique, we isolated about 50 bacterial strains from sediments of the Weser Estuary. A high percentage of bacteria among these isolates were capable of nitrate dissimilation or nitrite dissimilation. Quantitative determinations of nitrite-dissimilating bacteria have not been reported until now. It was therefore decided to determine the densities of the nitrate and nitrite-dissimilating bacteria in sediments of the Weser Estuary and the German Bight, in order to gain a better insight into the role of nitrite dissimilation in nitrogen transformation processes.

Materials and Methods

Sediment samples

Sediment samples were obtained with a Van Veen grab. After draining off the water column above the sediment, about 1 cm layer of the sediment surface was taken with a sterile spoon and filled into sterile 250 ml wide-neck bottles.

Test media

The basal medium consisted of Bacto Nutrient Broth (DIFCO) with the addition of 17.6 g/l NaCl. This medium, abbreviated as NB in the text, was used to determine the Most Probable Numbers (MPN) of heterotrophic bacteria, whereas the addition of 0.5 % KNO_3 (NB +

KNO_3) or 0.2 % KNO_2 (NB + KNO_2) made possible the records of nitrate-dissimilating and of nitrate plus nitrite-dissimilating bacteria, respectively. The MPN obtained with a fourth medium containing NB, 0.5 % KNO_3 and 0.2 % KNO_2 (NB + KNO_3 + KNO_2) were compared with the MPN from NB + KNO_2. The media were filled in 9 ml portions in test tubes and in each test tube a Durham tube was inserted for the detection of gas formation. The media were sterilized at 121° C for 20 minutes in an autoclave. A scheme giving additional informations about the criteria used for evaluating the MPN of bacteria from the four different test media is presented below:

NB	turbidity →	Positive:	Heterotrophic bacteria
	gas formation →	negative:	Control for gas formation other than from denitrification
NB + KNO_3	nitrite determination →	positive:	Nitrate-reducing bacteria (nitrate assimilating, nitrate dissimilating and nitrate respiring groups)
	gas formation →	positive:	Nitrate-dissimilating bacteria. Gas formation from extracellular nitrite, an intermediate of nitrate reduction, by nitrite-dissimilating bacteria is theoretically possible
NB + KNO_2	gas formation →	positive:	Nitrate-dissimilating plus nitrite-dissimilating bacteria. Only nitrate-dissimilating bacteria tolerating 0.2 % KNO_2 were recorded here
NB + KNO_3 + KNO_2	gas formation →	positive:	Nitrate-dissimilating plus nitrite-dissimilating bacteria. Only nitrate-dissimilating bacteria tolerating 0.2 % KNO_2 or higher nitrite concentrations were recorded here

Dilution tube method

Five spoonfuls (5 x 1 ml) of the sediment sample were suspended in 45 ml of dilution medium and from this suspension serial dilutions were made. Another spoonful (1 ml) of the sediment sample was dried at 60° C for 3 days and the dry weight was subsequently determined. The dilution medium contained (g/l): NaCl 11.738; $MgCl_2 \cdot 6H_2O$ 5.305; Tween 80 0.5; Bacto-Agar 1.5. One ml aliquots of each dilution were used to inoculate the four different media (3 parallel tubes/dilution) on board the ship and the tubes were then incubated at 18° C for 6 days. Turbidities in NB, both nitrite determination and gas formation in Durham tubes in NB + KNO_3 (see TAN 1970), or only gas formation in NB + KNO_2 and in NB + KNO_3 + KNO_2 were evaluated. The MPN of bacteria were taken from the tables in STANDARD METHODS FOR THE EXAMINATION OF WATER AND WASTEWATER (1971) and subsequently the MPN were calculated on a sediment dry weight basis. Subtraction of the MPN for nitrate-dissimilating bacteria from the MPN of nitrate plus nitrite-dissimilating bacteria gives the MPN for nitrite-dissimilating bacteria. The first intermediate in both biochemical pathways of nitrate reduction, whether dissimilatory or assimilatory, is nitrite (PAYNE 1973). PAYNE (1973) subdivided the bacteria capable of reducing nitrate dissimilatively into nitrate respiring (NO_3 – NO_2) and denitrifying (NO_3 – NO_2 – NO – N_2O – N_2) groups. The term "nitrate-dissimilating" used here is therefore identical with "denitrifying" and nitrite-dissimilating bacteria are those bacteria not capable of reducing nitrate but nitrite to elementary nitrogen. By determining nitrite in NB + KNO_3, all bacteria belonging to the nitrate assimilating, denitrifying and nitrate respiring groups were recorded and the term "nitrate-reducing" bacteria was chosen here (see TAN and OVERBECK 1973).

Results

Sediments samples were taken during 2 cruises with the *R. V. Victor Hensen* on 14 September 1977 and on 22 November 1977. As seen in Fig. 1, the sediments originated from the Weser Estuary near Bremerhaven and farther seawards till the Isle of Helgoland in the German Bight. The stations 1, 3, 5 and 9 were visited twice. The type of sediments collected during the second visit was different from that collected during the first visit in stations 1 and 3, whereas it was similar during both the visits in stations 5 and 9.

The results of the bacterial density determinations are presented in Table 1. The MPN of heterotrophic bacteria ranged from 493 to 602094/g sediment dry wt. A high percentage of these heterotrophic bacteria were capable of reducing nitrate to nitrite (33 %–100 %). An exception was the number of nitrate-reducing bacteria of the station 19 with only 5.2 % of the heterotrophic bacteria. The MPN of nitrate-reducing bacteria at 7 stations exceeded the MPN of heterotrophic bacteria, but taking into account the lower and upper limits at 95 % confidence for 3 parallel

Table 1. Bacterial densities (MPN/g dry wt.) in sediments of the Weser Estuary and the German Bight. Stations 1-14 on 14 September 1977, Stations with asterix and stations 15-20 on 22 November 1977. Marks of the buoys according to International System A (1978).

Station	Location	Depth (m)	Sediment type	Heterotrophs (NB: turbidity)	Nitrate-reducing bacteria ($NB+KNO_3$:nitrite)	Nitrate-dissimilating bacteria ($NB+KNO_3$: gas)		Nitrate plus nitrite-dissimilating bacteria ($NB+KNO_2$: gas)	Nitrate plus nitrite-dissimilating bacteria ($NB+KNO_3+KNO_2$: gas)	Nitrite-dissimilating bacteria	
						MPN	Percent of heterotrophs			MPN	Percent of heterotrophs
1	buoy 54	8	silt	>20 397	>20 397	78	< 0.4	445	445	367	< 1.8
1*	buoy 54	9	gravel	35 564	21 812	1 849	5.2	356	356	(-1 493)	0
2	buoy 57	15	fine sand	> 6 620	> 6 620	5	<0.08	144	277	272	< 4.1
3	53^o 35' N 8^o 31' E	14	silt	>18 395	>18 395	156	< 0.8	2 508	38	2 352	<12.8
3*	53^o 35' N 8^o 31' E	17	coarse and fine sand	493	1 272	0	0	48	19	48	9.7
4	buoy 49	13	fine sand	7 646	7 646	0	0	10	3	10	0.1
5	buoy 48	11	fine sand	5 976	5 976	12	0.2	51	2	39	0.7
5*	buoy 48	13	fine sand	8 080	2 655	54	0.7	139	12	85	1.0
6	buoy 44	14	fine sand	6 986	2 921	15	0.2	27	2	12	0.2
7	buoy 38	13	fine sand	7 279	7 279	0	0	0	20	20	0.3
8	buoy 34	13	sand	2 408	> 5 759	2	0.08	8	5	6	0.2
9	buoy 30	18	sand	2 579	2 579	2	0.08	13	0	11	0.4
9*	buoy 30	18	fine sand	1 376	1 376	0	0	21	0	21	1.5
10	buoy 27	15	sand	894	6 557	0	0	0	2	2	0.2
11	buoy 23	15	sand	621	3 070	0	0	0	0	0	0
12	buoy 19	7	silt	>12 108	>12 108	4	< 0.03	4	0	0	0
13	buoy 15	23	fine sand	> 4 065	4 065	0	0	1	0	1	<0.03
14	buoy 9	19	coarse sand	1 571	> 7 201	0	0	0	0	0	0
15	buoy A/7	19	fine sand	4 512	2 232	0	0	2	0	2	0.04
16	buoy A/2	13	fine sand	1 208	1 260	0	0	0	0	0	0
17	Nordergründe	24	sandy silt	138 114	264 718	2	0.002	2	0	0	0
18	54^o 03' N 7^o 59' E	29	silt	48 087	48 087	10	0.02	78	10	68	0.1
19	54^o 06' N 7^o 59' E	30	silt	602 094	31 414	5	0.0008	30	122	117	0.02
20	buoy 3	10	fine sand	5 732	14 792	6	0.1	148	573	567	9.9

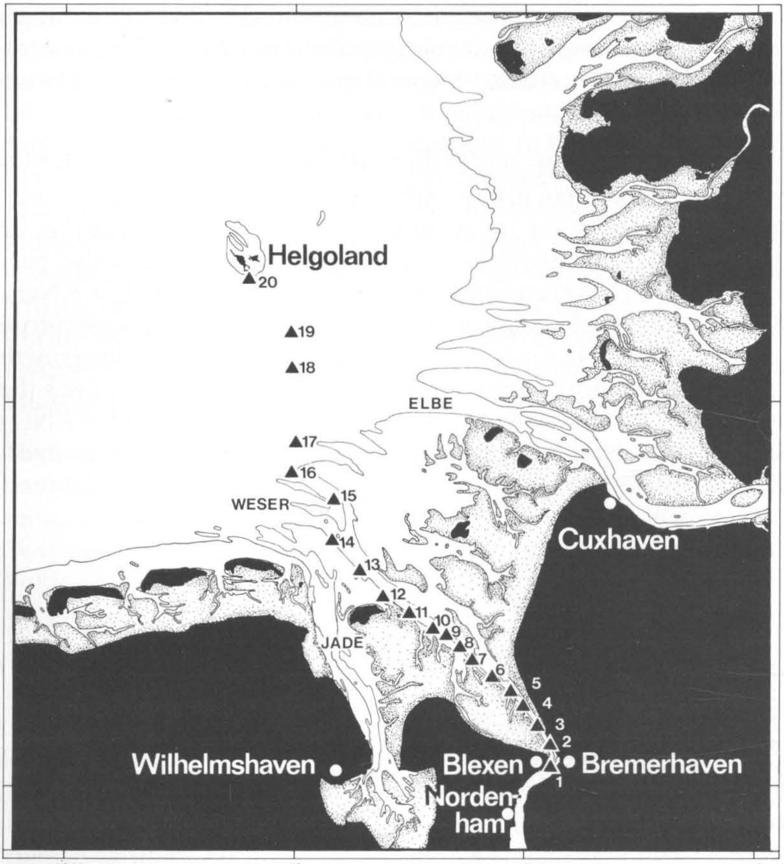

Fig. 1 Location of stations sampled in the Weser Estuary and in the German Bight

tubes per dilution, the true numbers of nitrate-reducing bacteria were not necessarily greater than the true numbers of the heterotrophic bacteria.

The numbers of nitrate-dissimilating bacteria were in the range of 0–156/g sediment dry wt. (up to 0.8 % of heterotrophic bacteria). An exception was the sediment of station 1 from 22 November 1977 with a MPN of 1849/g dry wt., or 5.2 % of the heterotrophs.

The amounts of nitrite-dissimilating bacteria were between 0 and 2352/g sediment dry wt. (up to 13 % of heterotrophic bacteria). Nearly all the numbers of nitrite-dissimilating bacteria were higher than the numbers of nitrate-dissimilating bacteria; only the stations 1 (from 22 November 1977), 6, 12 and 17 revealed higher amounts of nitrate-dissimilating bacteria.

The MPN of nitrate plus nitrite-dissimilating bacteria in NB + KNO_2 were always higher than just the MPN of nitrate-dissimilating bacteria, with the exception of the sediment sample from station 1 taken on 22 November 1977. The numbers of nitrate plus nitrite-dissimilating bacteria in NB+ KNO_2 were mostly higher than from NB + KNO_3 + KNO_2, with the exception of the stations 2, 7, 10, 19 and 20. The high nitrite concentrations used in the test media might have suppressed the growth of nitrate-dissimilating bacteria (WILLIAMS et al. 1978), and even the growth of nitrite-dissimilating bacteria could be inhibited in NB + KNO_3 + KNO_2, if higher nitrite concentrations - additionally formed as intermediate of nitrate reduction - occurred in the nutrient broth (VANGNAI and KLEIN 1974).

Discussion

The loss of nitrogen by denitrification processes in the marine environment was already the subject of many investigations (GOERING 1978). According to GOERING (1978) the annual rate of oceanic denitrification is about 3 x 10^{14}g (N)/yr. DUGDALE et al. (1977) and PACKARD et al. (1978) investigated the extent of bacterial nitrate reduction in the Peru upwelling region, whereas DEUSER et al. (1978) estimated the rate of denitrification in the Arabian Sea. In littoral (KOIKE and HATTORI 1978; SØRENSEN 1978) as well as in pelagic marine sediments (BENDER et al. 1977; WILSON 1978) the evidence for denitrification could be demonstrated. Nearshore sediments and bottom waters of estuaries and continental shelves with their relatively high organic carbon and low oxygen contents offer good conditions for denitrification. Although up to 50 % of the bacteria found in the sea are capable of reducing nitrate to nitrite, only a small number of them (0.01-5 %) are active denitrifiers (ZO BELL 1946). Higher percentages of denitrifying bacteria were also reported, as for instance from Red Sea sediments (HEITZER and OTTOW 1976).

A study of the reduction of nitrate and nitrite by marine bacteria was already conducted in 1901 by GRAN (see ZOBELL 1946). The role of nitrite-dissimilating microorganisms in nitrogen transformations is not clear. FOCHT and VERSTRAETE (1977) held the opinion that nitrite-dissimilating organisms are of minimal importance in most terrestrial and aquatic systems, where nitrite concentrations are generally low. After their studies of nitrite-dissimilating pseudomonads isolated from Oregon soils, VANGNAI and KLEIN (1974) were convinced that nitrite-dissimilating microorganisms might play an important role in nitrogen transformations in manured soil environments. In sediments of the Weser Estuary and the German Bight we found the numbers of nitrite-dissimilating bacteria mostly dominating over the numbers of nitrate- dissimilating bacteria. This phenomenon ist not yet known for other areas. VAN GYLSWYK (1961) also showed in his percentage estimation of denitrifying bacteria in sewage effluents that the number of nitrate plus nitrite-dissimilating bacteria (called nitrite reducing bacteria by VAN GYLSWYK) were sometimes higher than the number of nitrate-dissimilating bacteria (called nitrate reducing bacteria by him).

High internal concentrations of nitrite in cells of *Aerobacter aerogenes* and *Escherichia coli* were observed in a complex medium containing mineral salts solution, tryptone and 0.04 M nitrate (WIMPENNY and WARMSLEY 1968). The same authors also reported that internal accumulation of nitrite was not recorded with *Pseudomonas aeruginosa*, a denitrifying organism. However, up to 73 mg/l of nitrite-N were measured in the growth medium with *Pseudomonas aeruginosa*, grown anaerobically with nitrate as sole nitrogen source under hydrogen atmosphere (TAN 1973). If cultivated in nitrate-ammonium medium, *Pseudomonas aeruginosa* excreted only smaller amounts of nitrite (up to 213 μg/l of nitrite-N). It is therefore assumed that little nitrite was formed in NB + KNO_3 as an intermediate of denitrification activity.

In estimating the MPN of nitrate plus nitrite-dissimilating bacteria, 0.2 % KNO_2 was used in this study. The nitrite-dissimilating pseudomonads investigated by VANGNAI and KLEIN (1974) were capable of active dissimilation at a nitrite concentration of 3.642 g/l. Nitrate-dissimilating organisms however, could not tolerate nitrite concentrations as high as 0.2 % (BOVELL 1967; BOLLAG, ORCUTT and BOLLAG 1970; WILLIAMS et al. 1978). It is therefore possible that growth of nitrate-dissimilating bacteria was inhibited in NB + 0.2 % KNO_2 and MPN of nitrate plus nitrite-dissimilating bacteria could be higher, if a lower nitrite concentration is chosen.

The numbers of nitrate and nitrite-dissimilating bacteria showed a decreasing tendency down the estuary (station 1 to station 10). The highest numbers of these bacteria were found in the Weser off Bremerhaven and in the German Bight, south of the Isle of Helgoland (stations 18, 19 and 20). Organic pollution of the Weser Estuary with municipal sewage and industrial wastes from fishprocessing factories in Bremerhaven has been reported (Wachs 1971, 1973). The influence of the Elbe Estuary with its organic rich load, and wastewater pollution from the Isle of Helgoland might be responsible for the high numbers at the stations 18, 19 and 20. In such stations receiving a considerable amount of organic substances, high percentages of nitrite-dissimilating bacteria in relation to the numbers of heterotrophs were found. This was independent from the sediment type. A relationship with organic carbon contents of the sediment samples is quite possible. We therefore conclude from our results that in coastal sediments with high input of organic substrates, nitrate-dissimilating and nitrite-dissimilating bacteria are abundant and the low nitrite concentrations found in soil and aquatic environments might be related partially to the activity of the nitrite-dissimilating bacteria.

References

BENDER, M. L., K. A. FANNING, P. N. FROELICH, G. R. HEATH and V. MAYNARD (1977): Interstitial nitrate profiles and oxidation of sedimentary organic matter in the eastern equatorial Atlantic. Science, N.Y. **198:** 605-609.

BOLLAG, J. M., M. L. ORCUTT and B. BOLLAG (1970): Denitrification by isolated soil bacteria under various environmental conditions. Proc. Soil Sci. Soc. Am. **34:** 875-879.

BOVELL, C. (1967): The effect of sodium nitrite on the growth of *Micrococcus denitrificans*. Arch. Mikrobiol. **59:** 13-19.

CHATELAIN, R. (1969): Réduction des nitrites par *Alcaligenes odorans* var. *viridans*. Ann. Inst. Pasteur, Paris **116:** 498-500.

DEUSER, W. G., E. H. ROSS and Z. J. MLODZINSKA (1978): Evidence for and rate of denitrification in the Arabian Sea. Deep-Sea Res. **25:** 431-445.

DUGDALE, R. C., J. J. GOERING, R. T. BARBER, R. L. SMITH and T. T. PACKARD (1977): Denitrification and hydrogen sulfide in the Peru upwelling region during 1976. Deep-Sea Res. **24:** 601-608.

FOCHT, D. D. and W. VERSTRAETE (1977): Biochemical ecology of nitrification and denitrification. In M. ALEXANDER: Advances in microbial ecology. Volume 1. Plenum Press, New York, p. 135-214.

GOERING, J. J. (1978): Denitrification in marine systems. In D. SCHLESSINGER: Microbiology – 1978. American Society for Microbiology, Washington, p. 357-361.

HEITZER, R. D. and J. C. G. OTTOW (1976): New denitrifying bacteria isolated from Red Sea sediments. Mar. Biol. **37:** 1-10.

KOIKE, I. and A. HATTORI (1978): Denitrification and ammonia formation in anaerobic coastal sediments. Appl. Environ. Microbiol. **35:** 278-282.

PACKARD, T. T., R. C. DUGDALE, J. J. GOERING and R. T. BARBER (1978): Nitrate reductase activity in the subsurface waters of the Peru Current. J. Mar. Res. **36:** 59-76.

PAYNE, W. J. (1973): Reduction of nitrogenous oxides by microorganisms. Bacteriol Rev. **37:** 409-452.

PICHINOTY, F., J. BIGLIARDI-ROUVIER, M. MANDEL, B. GREENWAY, G. MÉTÉNIER and J. L. GARCIA (1976): The isolation and properties of a denitrifying bacterium of the genus *Flavobacterium.* Antonie van Leeuwenhoek **42:** 349-354.

PICHINOTY, F., M. VÉRON, M. MANDEL, M. DURAND, C. JOB and J.-L. GARCIA (1978): Etude physiologique et taxonomique du genre *Alcaligenes: A. denitrificans, A. odorans* et *A. faecalis.* Can. J. Microbiol. **24:** 743-753.

SØRENSEN, J. (1978): Capacity for denitrification and reduction of nitrate to ammonia in a coastal marine sediment. Appl. Environ. Microbiol. **35:** 301-305.

STANDARD METHODS FOR THE EXAMINATION OF WATER AND WASTEWATER (1971): Thirteenth edition. Amer. Public Health Ass. Inc. New York, 874 pp.

TAN, T. L. (1970): Ökologisch-physiologische Untersuchungen über nitratreduzierende Bakterien des Plußsees. Thesis Kiel University; 1-75.

TAN, T. L. and J. OVERBECK (1973): Ökologische Untersuchungen über nitratreduzierende Bakterien im Wasser des Plußsees (Schleswig-Holstein). Z. allg. Mikrobiol. **13:** 71-82.

TAN, T. L. (1973): Physiologie der Nitratreduktion bei *Pseudomonas aeruginosa.* Z. allg. Mikrobiol. **13:** 83-94.

VANGNAI, S. and D. A. KLEIN (1974): A study of nitrite-dependent dissimilatory microorganisms isolated from Oregon soils. Soil Biol. Biochem. **6:** 335-339.

VANGYLSWYK, N. O. (1961): Percentage estimation of denitrifying bacteria. J. biochem. microbiol. Technol. Engng. **3**: 115-117.

WACHS, B. (1971): Organisch verschmutzte Abwässer im Küsten- und Tidegebiet. Z. Wass. Abwass. Forsch. **4:** 105-117.

– (1973): Abwässer der Fischverarbeitungsindustrie im Küstenbereich. Z. Wass. Abwass. Forsch. **6:** 25-39.

WILLIAMS, D. R., J. J. ROWE, P. ROMERO and R. G. EAGON (1978): Denitrifying *Pseudomonas aeruginosa*: Some parameters of growth and active transport. Appl. Environ. Microbiol. **36:** 257-263.

WILSON, T. R. S. (1978): Evidence for denitrification in aerobic pelagic sediments. Nature, Lond. **274:** 354-356.

WIMPENNY, J. W. T. and A. M. H. WARMSLEY (1968): The effect of nitrate on krebs cycle enzymes in various bacteria. Biochim. biophys. Acta **156:** 297-303.

YUATT, J. B. (1954): Denitrification of nitrite by a species of *Achromobacter.* Nature, Lond. **173:** 826-827.

ZOBELL, C. E. (1946): Marine microbiology. A monograph on hydrobacteriology. Waltham, Mass., 240 pp.