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Influence of denitrification in aquatic sediments on the nitrogen content of natural waters

Abridged version of the thesis with the same title

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Abstract

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Forms part of a doctoral thesis, Wageningen ((vii) + 104 p., 43 figs, 28 tables). Other parts are published in Water Research, but are summarized in the Agric. Res. Rep.

A study was made of microbiological processes, particularly denitrification, leading to the elimination of nitrogen from natural waters. As denitrification is an anaerobic process and natural waters mostly contain dissolved oxygen, this process was suggested to proceed in the anaerobic sediment at the bottom of natural waters. Two widely differing types of aquatic sediments were tested in the laboratory for effects of temperature, oxygen and nitrate in the overlying water, and thickness of the sediment layer on the rate of denitrification. During disappearance of nitrate from the overlying water, by far most of the nitrate was converted to molecular nitrogen by denitrification and only a small part of the nitrate was utilized for cell synthesis (immobilization). Production of gases in the sediment was studied in the presence and absence of nitrate in the overlying water. The sequence detected during denitrification in sediments was $NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$. Oxygen and nitrate diffuse from the overlying water into the sediment. Therefore denitrification proceeded in the sediment below the layer where hydrogen donors were oxidized by oxygen. Redox potentials showed that denitrification shifted deeper into the sediment with time. Mainly heterotrophic denitrifying bacteria of the genera *Pseudomonas* and *Alcaligenes* were active in denitrification. Carbohydrates, acetic acid and sulphide were important hydrogen donors for denitrifying bacteria in aquatic sediment. The ultimate effect of denitrification in sediment for the nitrate content of natural waters was tested in an 800-m reach of canal below a discharge. The nitrate content of shallow natural waters decreased permanently and considerably. Two pieces of equipment were devised, allowing simultaneous measurements of the uptake of oxygen and nitrate by completely mixed suspensions of sediments and undisturbed sediment cores.

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The author graduated on 8 October 1976 as Doctor in de Landbouwwetenschappen at the Agricultural University, Wageningen, the Netherlands, on a thesis with the same title.

Contents

1 General introduction

Maintaining a good quality of natural waters in the Netherlands is of vital importance, because (a) natural waters are increasingly used for preparation of drinking water, the demand for which is steadily increasing and (b) the dense population and its activities require the availability of suitable waters for agriculture, industry and recreation. However, the natural waters in the Netherlands, being already fairly rich in nutrients by nature, are tending to become seriously enriched with inorganic nutrients (eutrophication) as a result of the rapidly increased amount of pollution from the human society. Under such conditions, natural biological equilibria existing in natural water can easily be disturbed. Often excessive populations of algae develop. Such algal blooms may seriously affect the quality of natural water by consumption of oxygen by the algae themselves at night and by bacteria thriving on the algal material. These activities may even cause anaeroby, killing fish and causing deterioration of the taste of the water. Such adverse characteristics may seriously harm the suitability of natural waters for use. Efforts have thus to be made to stop eutrophication.

As algae are using light for energy supply and inorganic nutrients for cell synthesis, growth of these micro-organisms in natural waters can be limited by minimizing at least one of the growth factors in effluents being discharged into natural waters. Nitrogen and phosphorus are known to be suitable growth-limiting factors.

At present, purification of domestic and industrial waste water is mostly restricted to the reduction in biological oxygen demand. The inorganic nutrients, including nitrogen and phosphorus, often remain in the effluent in sufficiently large amounts to allow large-scale growth of algae when the effluent is discharged into natural water. Methods for removing nitrogen and phosphorus from effluents have already been developed. Introducing these methods into the existing purification systems usually requires high investment. In farming fertilizers are increasingly needed to raise food production as the world population increases. Drainage water from arable land may contain relatively high concentrations of nitrate, so that discharge of drainage water into surface waters is considered to be a substantial contribution to eutrophication.

In the present study, investigations were made on the influence of naturally occurring denitrification on the nitrogen content of natural water loaded with nitrogen-containing effluent or drainage water. Natural waters generally contain dissolved oxygen in such a concentration that denitrification in the water phase is practically impossible. Therefore, natural sediment with overlying water was chosen as a model system for laboratory experiments. Firstly, the factors affecting the rate of denitrification in water-sediment systems were tested (Chapter 2). Microorganisms involved in denitrification immobilize a certain amount of nitrogen in cell synthesis. The immobilized nitrogen can be derived from nitrate or from other nitrogen sources present in the sediment. A large part of the

immobilized nitrogen is mineralized after some time, whereupon the nitrogen moves largely into the overlying water and becomes available as nutrient for the growth of algae. Immobilization of nitrate nitrogen is unfavourable compared with denitrification. Therefore, it was investigated which part of the nitrate disappeared from the overlying water by denitrification and which part by immobilization (Chapter 3). A study was made of the influence of nitrate upon the production pattern of various gases formed in the sediment (Chapter 4). During denitrification, the available hydrogen donors in the upper layer of sediment become exhausted, resulting into a shift of the zone of denitrification to a deeper layer. The course of this shift as a function of time has been studied indirectly in Chapter 5. The results of the microbiological examination of a sediment with special attention to denitrifying bacteria are presented in Chapter 6. The availability of hydrogen donors for denitrifying bacteria in sediment has been studied in Chapter 7. The ultimate practical effect of the denitrification process on the total amount of nitrogen in surface water has been tested in a field experiment (Chapter 8). Finally, two pieces of equipment in denitrification studies are described, allowing simultaneous measurements of uptake of oxygen and nitrate by completely mixed suspensions of sediments and undisturbed sediment cores (Chapter 9).

2 Factors affecting the denitrification rate in two water-sediment systems

Abstract

Effects of temperature, oxygen and nitrate concentrations of the overlying water, and the thickness of the sediment layer on the rate of denitrification in the sediment were investigated in two water-sediment systems, A and B. At 4°C, denitrification started after a prolonged lag period in contrast to nitrification which did not occur significantly. At 15°C, and particularly at 25°C, both processes proceeded readily. The disappearance of NO_2^--N from the overlying water was more rapidly than that of NO_3^--N.

The denitrification rate was slightly reduced by increasing the dissolved oxygen concentration in the overlying water from 0 to approximately 2 mg l^{-1}. A further rise of the dissolved oxygen concentration had no further decreasing effect on the denitrification rate.

The denitrification rate in sediment was dependent on the nitrate concentration in the overlying water approximating first order kinetics at lower concentrations, gradually becoming independent of the nitrate concentration at higher nitrate contents (zero order kinetics).

When starting with a nitrate-nitrogen concentration of 25.2 mg l^{-1}, a sediment layer of 7 mm with A and 14 mm with B was roughly found to be involved in denitrification.

Denitrification rates found in the present laboratory experiments were supposed to be considerably lower than those occurring under natural conditions as additional mechanisms for the transport of nitrate into sediments occurred in natural environments.

3 The immobilization of nitrogen in a water-sediment system by denitrifying bacteria as a result of nitrate respiration

Abstract

The immobilization of nitrate and ammonium nitrogen by denitrifying bacteria upon the addition of nitrate to an anaerobic water-sediment system was studied under laboratory conditions. Two sediments were used originating from a ditch on which effluent of a purification plant for liquid animal manure is discharged and from a ditch on which water from arable land is drained off, respectively. The former (sediment A) contained 3.36% organic matter on a dry weight basis, the latter (sediment B) 1.37%. Most of the added nitrate was lost by volatilization due to denitrification, *viz.* 97.2% in sediment A and 94.5% in sediment B. The remaining part, 2.8 and 5.5%, respectively was converted into organic matter and ammonia. In addition to nitrate nitrogen, ammonium nitrogen was immobilized by the denitrifying bacteria during the nitrate respiration. The quantities of immobilized ammonium nitrogen derived from sediments A and B appeared to be equal to 7.3 and 4.1%, respectively when calculated on the added nitrate nitrogen. The total amount of nitrogen (NO_3^--N + NH_4^+-N) immobilized due to the addition of nitrate was equal to approximately 10% of the nitrate nitrogen added for both sediments. The relatively high percentage of added nitrate immobilized in sediment B may have been due to its relatively low ammonium content as compared to that of sediment A.

4 Gas production in aquatic sediments in the presence and absence of nitrate

Abstract

Experiments have been carried out with two anaerobic water-sediment systems, A and B, widely differing in organic matter content. A description was given of the apparatus used for measuring gas production in these systems in the presence or absence of added nitrate. For measuring He, N_2, O_2 and NO an improved gas-chromatographic separation was applied. The nitrate, nitrite and ammonium contents of the overlying water and the N_2, NO, N_2O, CH_4 and CO_2 contents of the gas phase were determined during incubation at 25°C in the presence and absence of 50 mg nitrate nitrogen. The sequence of the different nitrogenous compounds detected during denitrification in the sediment appeared to be: $NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$. There was a correlation between the rates of denitrification and methane formation on the one hand and the availability of the organic matter in the sediment on the other hand. In the presence of nitrate the black colour of sediment A turned partly grey-brown, while the black colour of sediment B turned entirely yellow-brown. From the amount of faded sediment and from the course of the nitrate curves in the overlying water it was concluded that in sediment A the exhaustion of nitrate became the limiting factor of denitrification and in sediment B the depletion of available organic matter. In sediment B nitrate was shown to suppress the formation of methane. Without added nitrate the rate of CH_4 formation at 25°C of sediment A was 0.98 and that of sediment B 0.04 μmoles per g dry weight of sediment per day.

5 The relation between redox potential and denitrification in a water-sediment system

Abstract

In the overlying water of a water-sediment system the pH was controlled at 7.0, the nitrate-nitrogen concentration at 25.0 mg l^{-1} and the dissolved oxygen concentration above 6.1 mg l^{-1}. The temperature of the whole system was kept at 15°C. The average rate of nitrate removal from the system as a result of denitrification amounted to 160 mg NO_3^--N m^{-2} day^{-1}. By means of Eh measurements at various depths in the sediment, it was attempted to figure out the course of the penetration fronts of nitrate and oxygen in the sediment during 241 days of incubation. From these results the layer in which denitrification occurred was derived. The course of the denitrification zone was followed during the incubation period. As a result of the depletion of the available hydrogen donors in the sediment, oxygen reached the bottom of the sediment after 235 days of incubation.

6 Microbiological examination of an aquatic sediment with special attention to the population of denitrifying bacteria

Introduction

In a previous investigation (Chapter 2), it was shown that nitrate is removed from natural waters containing dissolved oxygen by denitrification in the anaerobic sediments. A large number of bacterial species belonging to various genera have been reported (Payne, 1973) to be capable of reducing nitrate dissimilatory to nitrite only (nitrate reduction) or to gaseous products like nitrous oxide or molecular nitrogen (denitrification).

The denitrifying bacteria are heterotrophs except *Thiobacillus denitrificans* which is a chemoautotroph as it derives energy from the oxidation of reduced forms of inorganic sulphur compounds and fixes carbon dioxide for synthesis of cell material (e.g. Aminuddin & Nicholas, 1973; Baldensperger & Garcia, 1975). However Tuttle & Jannasch (1972) reported that chemoautotrophic thiobacilli like *T. denitrificans* occur only rarely in the marine environment. They found that the biological oxidation of reduced sulphur compounds in open sea areas is largely carried out by facultatively autotrophic bacteria. Most of their isolates grew anaerobically with nitrate. Tuttle *et al.* (1974) described three strains of marine heterotrophic bacteria which can denitrify with both organic compounds and thiosulfate as hydrogen donors. Besides denitrifying bacteria that utilize nitrate or nitrite, specialized nitrite-denitrifying bacteria have recently been isolated (Pichinoty & Chatelain, 1973; Vangnai & Klein, 1974). They were incapable of reducing nitrate. A new field in denitrification studies was claimed by Davies (1973), who isolated *Alcaligenes, Achromobacter, Pseudomonas* and *Bacillus* species, that would be able to utilize methane as hydrogen donor during denitrification.

Vanderpost (1972) analysed sediments of Lake Ontario for total aerobic and anaerobic heterotrophic bacteria, sulphur-oxidizing, sulphate-reducing, ammonifying, nitrogen-fixing and denitrifying bacteria during 8 months in an attempt to demonstrate seasonal variations in these counts. She concluded that the variations observed in the bacterial populations were independent of the seasons. Dutka *et al.* (1974) examined offshore Lake Erie sediments for total heterotrophs and ammonifying, nitrifying, nitrate-reducing, denitrifying, sulphur-oxidizing, sulphate-reducing, organic sulphur-reducing, iron-oxidizing, and insoluble organic and inorganic phosphate-solubilizing bacteria.

The aim of this study was the bacteriology of a shallow freshwater sediment with special attention to denitrifying bacteria.

Materials and methods

Sediment

Experiments were with sediment from a ditch into which effluent was discharged from a purification plant. This plant consisted of an aeration tank for activated sludge and a final settling tank. The plant was loaded with slurry from a veal production unit. Sediment of the same origin was used in previous investigations (Chapters 2, 3, 4 and 5).

Media

Heterotrophic denitrifying bacteria (HD) were counted and isolated on a low-nutrient medium (HD). As a preliminary, various media reported in the literature were tested. HD medium was selected as the highest counts were obtained with the tested sediment. HD medium contained: sodium lactate, 1 g; glycerol, 0.5 g; yeast extract, 0.2 g; Casamino-acids, 0.2 g; beef extract, 0.1 g; K_2HPO_4, 0.25 g; $MgSO_4.7H_2O$, 1 g; $CaCl_2$, 0.3 g; $FeSO_4.7H_2O$, 0.01 g; ferric ammonium citrate, 0.01 g; NH_4Cl, 0.1 g; KNO_3, 2 g; vitamin B_{12}, 5 μg; soil extract, 100 ml; minor elements solution, 1 ml; tap water, 900 ml; pH, 7.0. For solid media 12 g l^{-1} agar (Oxoid) was added. The minor elements solution was prepared according to Pochon & Tardieux (1962) and contained K_2MoO_4, 0.05 g; $Na_2B_4O_7$, 0.05 g; $FeCl_3$, 0.05 g; $Co(NO_3)_2.6H_2O$, 0.05 g; $CdSO_4$, 0.05 g; $CuSO_4$, 0.05 g; $ZnSO_4$, 0.05 g; $MnSO_4$, 0.05 g; distilled water, 1000 ml. The test for growth of the heterotrophic denitrifying bacteria on various organic substrates was with a basal medium (HDB) composed of: Casamino-acids, 0.1 g; yeast extract, 0.01 g; K_2HPO_4, 0.25 g; NH_4Cl, 0.2 g; $MgSO_4.7H_2O$, 0.1 g; $CaCl_2$, 0.3 g; $FeSO_4.7H_2O$, 0.01 g; ferric ammonium citrate, 0.01 g; vitamin B_{12}, 5 μg; minor elements solution, 1 ml; soil extract, 100 ml; tap water, 900 ml; pH, 7.0.

The medium for isolation of the autotrophic denitrifying bacterium, *Thiobacillus denitrificans* (TD medium), was prepared according to Baalsrud & Baalsrud (1954). It contained: $Na_2S_2O_3.5H_2O$, 5 g; KNO_3, 2 g; NH_4Cl, 0.5 g; $MgSO_4.7H_2O$, 0.8 g; $FeSO_4.7H_2O$, 0.01 g; $NaHCO_3$, 1 g; KH_2PO_4, 2 g; minor elements solution, 1 ml; vitamin B_{12}, 5 μg; distilled water, 1000 ml; pH 7.0. To solidify the medium, 15 g l^{-1} specially purified agar (DIFCO Laboratories) was added.

Aerobic heterotrophic bacteria were grown on a low-nutrient medium (AH), containing glycerol, 0.2 g; glucose, 0.2 g; yeast extract, 0.2 g; asparagine, 0.01 g; calcium acetate, 0.01 g; NH_4Cl, 0.01 g; KH_2PO_4, 0.1 g; $FePO_4$, 0.01 g; $MgSO_4.7H_2O$, 0.01 g; minor elements solution, 1 ml; soil extract, 500 ml; tap water, 500 ml; agar (Oxoid), 10 g; pH, 7.0.

Anaerobic heterotrophic bacteria were grown in ANH medium (General laboratory procedures), which contained: peptone, 10 g; glycerol, 10 g; yeast extract, 1 g; K_2HPO_4, 0.5 g; agar (Oxoid), 12 g; tap water, 1000 ml; pH, 7.0.

Ammonifying bacteria were cultured in AVM medium (Pochon & Tardieux, 1962), which contained: asparagine, 0.2 g; Winogradsky solution, 50 ml; minor elements solution, 1 ml; distilled water, 950 ml; pH, 7.0. Winogradsky solution contained: K_2HPO_4, 5 g; $MgSO_4$, 2.5 g; NaCl, 2.5 g; $Fe_2(SO_4)_3$, 0.05 g; $MnSO_4$, 0.05 g; distilled water, 1000 ml.

Counts of nitrifying bacteria were on two media. Medium NSPT (Pochon & Tardieux, 1962) for *Nitrosomonas* spp., contained: $(NH_4)_2SO_4$, 0.5 g; $CaCO_3$, 1 g; Winogradsky solution, 50 ml; distilled water, 950 ml. Medium NBPT (Pochon & Tardieux, 1962), for cultivating *Nitrobacter* spp., contained: $NaNO_2$, 1 g; $CaCO_3$, 1 g; Winogradsky solution, 50 ml; distilled water, 950 ml.

All media were autoclaved at 120 °C for 20 min.

Occurrence of bacteria belonging to various physiological groups at different depths in a sediment core

A sediment core was taken from the ditch with a Jenkin mud sampler (Mortimer, 1942). In the laboratory, the overlying water of the sediment was sucked off, after which a sample of 1 g wet sediment was taken at different depths: 0.5, 2, 5, 10 and 15 cm in the sediment. Each sample was mixed in a culture tube with 9 ml sterile solution containing sodium chloride 9 g l^{-1} and sodium pyrophosphate 1 g l^{-1} for 3 min. Subsequently, a decimal dilution series with sterile sodium chloride solution 9 g l^{-1} was prepared. Plates and tubes were inoculated for the enumeration of aerobic and anaerobic heterotrophic bacteria, denitrifying bacteria, ammonifying bacteria and nitrifying bacteria.

Aerobic heterotrophic bacteria per gram of wet sediment at a certain depth were counted by the spread-plate technique. Two dried plates with AH medium were used for each dilution. The plates were incubated aerobically at 25°C for 7 days.

Anaerobic heterotrophic bacteria were counted in closed culture tubes containing ANH agar medium. After sterilization, two tubes per dilution were inoculated at 45°C. After mixing and cooling, the solidified media were covered with a 15 mm layer of sterile vaspar and incubated at 25°C during 14 days.

Denitrifying bacteria were counted by means of the Most Probable Number (MPN) method. Three tubes, each containing 12 ml HD medium and a Durham tube, were employed for each dilution. The tubes were incubated at 25 °C in an anaerobic jar, using the GasPak system described by Ferguson *et al.* (1975), for 14 days. Denitrification was examined by a modified method derived from that of Focht & Joseph (1973) and Patriquin & Knowles (1974). Turbid tubes were checked for the absence of nitrate by the diphenylamine-sulphuric acid reagent described by Pochon & Tardieux (1962). As confirmation, the produced gas was collected in Durham tubes. Carbon dioxide in the Durham tubes was removed by adding a few drops of concentrated sodium hydroxide. The number of denitrifying bacteria was established from the MPN table (Standard Methods, 1971).

Ammonifying bacteria were also counted by the MPN method using AVM medium. Three tubes were incubated aerobically at 25°C for 14 days. Then the ammonium production was checked with a few drops of Nessler's reagent (Deutsche Einheitsverfahren, 1960).

Nitrifying bacteria were enumerated as *Nitrosomonas* spp. and *Nitrobacter* spp. by the MPN technique with NSPT and NBPT medium, respectively. The tubes were closed with metal caps instead of cotton-wool plugs to prevent contamination of the medium with organic substances from the plugs during sterilization. The tubes were incubated aerobically at 25°C for 28 days. Thereafter the presence of both nitrite and nitrate in the cultures of *Nitrosomonas* spp. and the presence of nitrate in those of *Nitrobacter* spp.

were checked with two tests described by Pochon & Tardieux (1962).

The sampling of the sediment core, the preparing of the dilution series with samples from different depths in the sediment and the inoculation of both the plates and the culture tubes were carried out within 8 h. All of the experiments described in this section have been carried out with two cores taken in June and August 1975, respectively.

Enumeration and identification of the denitrifying population in sediment under field conditions and after anaerobic incubation with nitrate under laboratory conditions

The upper 5-cm layer of a sediment core taken from the ditch was homogenized. Part of this sediment was analysed chemically. One gram of wet sediment was introduced into each of 12 culture tubes containing 9 ml water.

Two of these tubes were used for preparing two decimal dilution series. Spread plates were prepared in duplicate from both dilution series. Plates with HD and TD medium with and without nitrate were incubated in an anaerobic jar as described earlier. The colonies on plates of the four treatments were counted after 20 days at 15°C. Thereafter, 100 colonies were picked off at random from a plate with HD medium and 50 colonies from a plate with TD medium, which had both been incubated anaerobically in the presence of nitrate.

The 10 remaining sediment-containing tubes were supplied with 2.5 mg nitrate nitrogen per tube and incubated in the dark at 15°C in an anaerobic jar. Twice a week the overlying water of the sediment of one tube was analysed for nitrate by the salicylate method described by Müller & Widemann (1955). If necessary, the amount of nitrate nitrogen in the remaining tubes was made up to 2.5 mg, whereafter the experiment was continued. Incubation was stopped when nitrate consumption was nearly nill. Then, two of the remaining tubes were used for a similar enumeration and isolation procedure as described above.

All strains isolated from the HD medium were tested for denitrification and nitrate reduction. For that purpose each isolate was inoculated in two tubes, each containing 12 ml HD medium and a Durham tube, and incubated at 25°C in an anaerobic jar for 14 days. Denitrification was checked by the method described on p. 47. Isolates that could not denitrify were examined for nitrate reduction by testing for the formation of nitrite with Griess-Romijn van Eck reagent (NEN 3235 6.3, 1972). Denitrifying and nitrate-reducing strains were identified with the scheme of Schmidt-Lorenz (1965). Doubtful issues in the identification test were checked with 'A guide to the identification of the Genera of Bacteria' (Skerman, 1967) and 'Bergey's Manual of Determinative Bacteriology' (1974). For maintenance, the strains were grown aerobically on agar slopes containing HD medium without nitrate. Strains isolated on TD medium that reduced nitrate or denitrified were tested in duplicate for aerobic heterotrophic growth on AH medium at 25°C.

Growth of heterotrophic denitrifying isolates on hydrogen donors

Utilization of various organic hydrogen donors by 13 heterotrophic isolates was tested in 100-ml Erlenmeyer flasks containing 25 ml HDB medium supplied with sterile substrate solutions to a final concentration of 1 g l^{-1}. The organic hydrogen donors tested

included: cellulose (snips of Whatman filter paper no. 4), cellobiose, xylan (Fluka AG), pectin, galacturonic acid, galactose, mannose, glucose, xylose, arabinose, sodium acetate and sodium lactate. The Erlenmeyer flasks were incubated aerobically at 25 °C on a rotary shaker. The cultures were examined for growth after 28 days. Those with cellulose were incubated again and examined after another 32 days. All these tests were carried out in duplicate.

Oxidation of thiosulphate to tetrathionate by heterotrophic denitrifying bacteria

The same 13 strains were also tested for their ability to oxidize thiosulphate to tetrathionate. Three hundred ml Erlenmeyer flasks with 100 ml HD medium supplemented with $Na_2S_2O_3.5H_2O$ 5 g l^{-1} were inoculated and incubated aerobically at 25°C for 15 days. Thereafter, the cell suspension was centrifuged and the clear supernatant was tested for tetrathionate by the method of Nietzel & Desesa (1955). This test was in triplicate.

Results and discussion

Occurrence of bacteria belonging to various physiological groups at different depths in a sediment core

The appearance of both sediment cores was similar. A layer of soft watery dark brown mud was present to about 1 cm below the water-sediment interface. From 1 to 15 cm below the interface, the cores consisted of a highly reduced black sandy layer which gradually turned dark grey.

The meaning of the values for bacteria of a distinct physiological group at a particular depth is questionable, but comparison of values at various depths tested indicates the distribution of that group throughout the sediment core. The distribution of aerobic and anaerobic heterotrophic bacteria, denitrifying bacteria, ammonifying bacteria, and nitrifying bacteria in the sediment core, as estimated in August 1975, is presented in Figure 6.1. An almost identical pattern had been found in the sediment core of June 1975. The highest counts of all physiological groups tested were found in the sediment at a depth of about 2 cm.

A comparison of the counts of different groups of bacteria at one particular depth in the sediment was not permissible because of the use of different counting procedures. However counts at all depths indicate trends in the ratios of bacteria of different groups. For that reason, the ratio of aerobic to anaerobic heterotrophs counted at all test depths was calculated (Table 6.1). These data show that no significant trend with depth existed in contrast to the results of Vanderpost & Dutka (1971) and Vanderpost (1972), who observed that anaerobic bacteria constituted a larger proportion of the total heterotrophic population with increased depth, because the numbers of anaerobic bacteria decreased less with depth than those of aerobes.

The highest count of heterotrophic denitrifying bacteria was at a depth of 2 cm (Fig. 6.1). This count decreased with increasing depth. A similar trend is seen when counts of heterotrophic denitrifiers were calculated as a percentage of total aerobic heterotrophs (Fig. 6.2). These results agree with those calculated from enumeration data

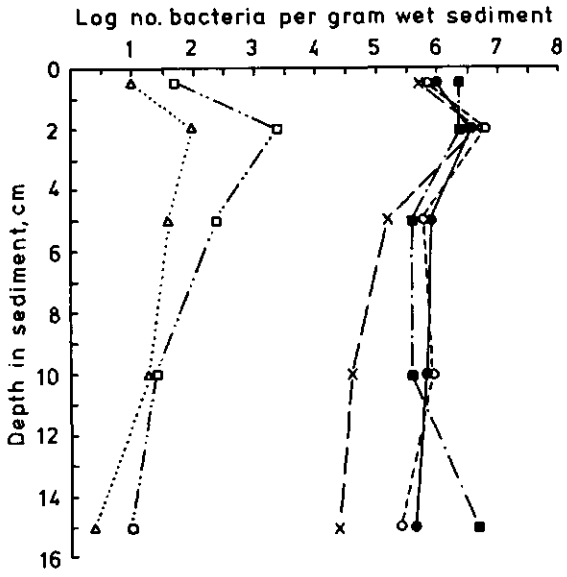

Fig. 6.1. Numbers of aerobic and anaerobic heterotrophic bacteria, and of denitrifying, ammonifying and nitrifying bacteria in a sediment core (August, 1975). ●———● Aerobic heterotrophs; ○- - -○ Anaerobic heterotrophs; X— —X Heterotrophic denitrifiers; ■——.——■ Ammonifying bacteria; □–..–□ *Nitrosomonas* spp.; △.....△ *Nitrobacter* spp.

Table 6.1. Ratio of numbers of aerobic to anaerobic heterotrophic bacteria at 0.5, 2, 5, 10, and 15 cm depth in the sediment (Data for August 1975).

Depth (cm)	Aerobes/anaerobes
0.5	1.4
2	0.5
5	1.3
10	0.9
15	1.8

of sediments of Lake Ontario and Lake Erie reported by Vanderpost (1972) and Dutka *et al.* (1974), respectively. From the results shown in Figures 6.1 and 6.2, it was concluded that the highest denitrifying activity occurred in the upper sediment layer.

The relatively high counts of ammonifying bacteria in the sediment in relation to the total population indicates the presence of a high content of organic nitrogenous compounds.

Nitrosomonas spp. and *Nitrobacter* spp. constituted only a small part of the total population in the sediment. The occurrence of nitrifying bacteria in anaerobic layers of sediment was surprising; it was probably a result of transport of these bacteria from the water-sediment interface into deeper layers of sediment by 'mixing' in sediments (Lee,

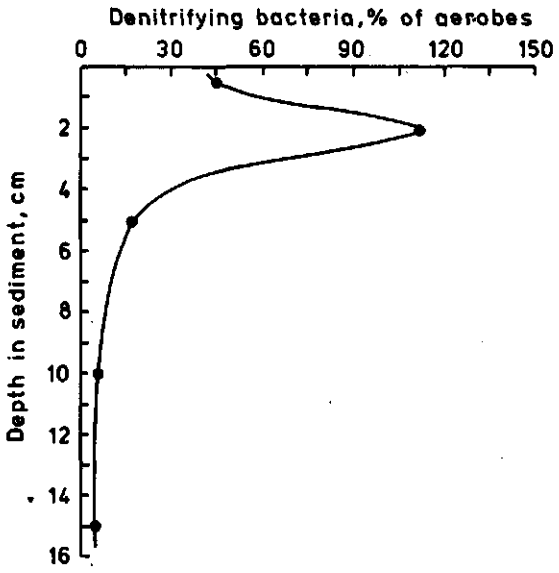

Fig. 6.2. Numbers of heterotrophic denitrifying bacteria as percentage of the numbers of aerobic heterotrophs in relation to depth in the sediment.

1970; Chapter 2). Laudelot *et al.* (1968) observed a fair resistance of nitrifying bacteria to anaerobic conditions. Similar counts and distribution patterns of nitrifying bacteria were observed in sediments of Lake Ontario (Vanderpost, 1972).

Enumeration and identification of the denitrifying population in sediment under field conditions and after anaerobic incubation with nitrate under laboratory conditions

Some chemical data on the sediment used are listed in Table 6.2.

For the enumeration and isolation of denitrifying bacteria, using the HD and TD media, an incubation temperature of 15°C was chosen, because this temperature has frequently been measured in the sediment. The 10 tubes with 1 g wet sediment and 9 ml solution containing 2.5 mg nitrate nitrogen were incubated anaerobically in the dark to eliminate growth of phytoplankton and photosynthetic sulphide-oxidizing bacteria. The amount of nitrate nitrogen consumed by the organisms in 1 g wet sediment is presented cumulatively in Figure 6.3. The consumption rate of nitrate in the sediment increased

Table 6.2. Some chemical data (mg per g dry weight) of the sediment used for counting and identification of the denitrifying population.

Water content	420
Organic matter determined as volatile solids	30.7
Ammonium nitrogen	0.38
Organic nitrogen	1.26
pH	7.5

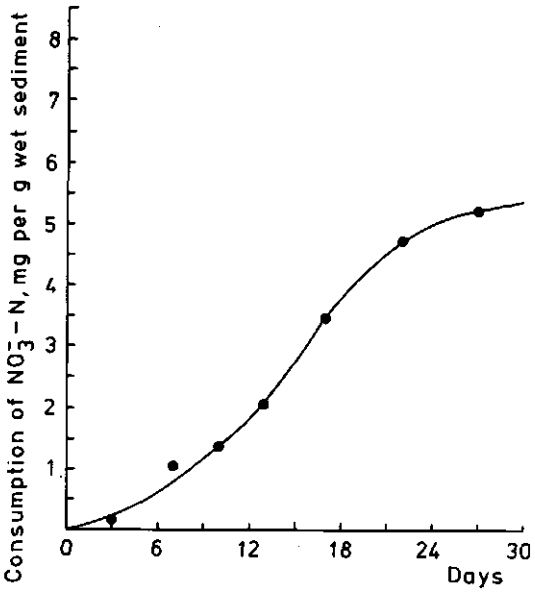

Fig. 6.3. Amount of nitrate nitrogen consumed by 1 g of wet sediment during incubation at 15°C.

gradually with incubation time up to 15 days, whereafter it declined slowly to a low constant value (endogenous nitrate respiration). Apparently, the pool of available hydrogen donors for denitrification had gradually been exhaused after 24 days, so that denitrifying organisms failed to increase. To identify the denitrifying bacteria responsible for the nitrate consumption in the tubes, a similar procedure for counting and isolation to that performed at the beginning of the experiment was started on the 28th day. The counts on HD medium under field conditions (at the start of the experiment) and after anaerobic incubation of the sediment in the presence of nitrate under laboratory conditions are given in Table 6.3. During the anaerobic incubation of the sediment in the presence of nitrate, the heterotrophic denitrifying bacteria increased to a larger extent than the facultative and obligately anaerobic bacteria (Table 6.3), presumably because the denitrifying bacteria derived more energy from the substrate by oxidative phosphorylation than the fermenting bacteria by substrate-level phosphorylation.

Table 6.3. Counts of heterotrophic bacteria per g wet sediment under field conditions (before incubation) and after anaerobic incubation with nitrate at 15°C under laboratory conditions. During this incubation, hydrogen donors from the sediment were utilized. Bacteria were counted on HD medium under anaerobic conditions in the presence or absence of nitrate.

Time of counting	Counts on plates	
	with NO_3^-	without NO_3^-
Before incubation	0.99×10^6	0.06×10^6
After incubation	233×10^6	2.70×10^6

Identification of heterotrophic denitrifying population from the sediment under field conditions was performed by picking off 100 colonies from an anaerobically incubated counting plate with nitrate-containing HD medium. It was expected that about 94% of the colonies on this plate consisted of nitrate-reducing and denitrifying bacteria (Table 6.3). Actually, 71 isolates were obtained in pure culture, the remaining strains being lost during isolation. Of these 71 strains, 50 denitrified and 19 other strains reduced nitrate only to nitrite. These 69 strains belonged to the genera listed in Table 6.4.

Heterotrophic denitrifying population occurring in the sediment after anaerobic incubation in the presence of nitrate under laboratory conditions was identified as described above. In this case, about 99% of the colonies picked off the counting plate consisted of nitrate-reducing and denitrifying bacteria (Table 6.3). Of the 92 isolates obtained in pure culture, 89 isolates denitrified and 3 isolates reduced nitrate to nitrite only. Results of the identification of this group of bacteria are presented in Table 6.5.

Table 6.4. Heterotrophic denitrifying bacterial population in sediment under field conditions.

Genera	Numbers of isolates		
	total	nitrate-reducing[a]	denitrifying
Pseudomonas	23	3	20
Alcaligenes	22	8	14
Bacillus	9	5	4
Flavobacterium	6	0	6
Xanthomonas	3	1	2
Cytophaga	2	1	1
Vibrio	2	0	2
Propionibacterium	1	0	1
Staphylococcus	1	1	0
Total number	69	19	50

[a] Strains reducing nitrate to nitrite only.

Table 6.5. The heterotrophic denitrifying population in sediment after anaerobic incubation in the presence of nitrate under laboratory conditions at 15°C.

Genera	Numbers of isolates		
	total	nitrate-reducing[a]	denitrifying
Pseudomonas	72	0	72
Alcaligenes	15	0	15
Bacillus	1	1	0
Vibrio	2	2	0
Micrococcus	1	0	1
Spirillum	1	0	1
Total number	92	3	89

[a] Strains reducing nitrate to nitrite only.

The population of the denitrifying bacteria in the sediment under field conditions consisted of 8 genera, of which *Pseudomonas, Alcaligenes, Bacillus* and *Flavobacterium* occurred most frequently, whereas after anaerobic incubation with nitrate, the population consisted of 4 different genera, of which *Pseudomonas* and *Alcaligenes* were by far the most numerous. It is likely that the population found after anaerobic incubation had really been involved in denitrification under field conditions. This explains also the higher ratio of nitrate-reducing to denitrifying strains in sediment under field conditions (Table 6.4) than after anaerobic incubation in the presence of nitrate under laboratory conditions (Table 6.5). These tables show that the denitrifying *Bacillus* species found in the sediment under field conditions had not really been involved in the denitrification process. This agrees with the observation of Woldendorp (1963) that in grassland sods the population of organisms with denitrifying capability consisted almost exclusively of *B. cereus*, whereas after application of nitrate to the sods, *Pseudomonas* spp., *Alcaligenes* spp. and *B. macerans* were mainly involved in denitrification.

Counts on TD medium under field conditions and after anaerobic incubation of the sediment in the presence of nitrate are given in Table 6.6. They show that during anaerobic incubation with nitrate a pronounced increase in nitrate-consuming bacteria had occurred. Of the 50 colonies picked off the TD plates inoculated with sediments before and after anaerobic incubation with nitrate, 2 and 7 strains, respectively, grew only autotrophically and as a consequence belonged to the genus *Thiobacillus*. As these organisms reduced nitrate only to nitrite, they were not identical with *T. denitrificans*. This result pointed to the absence of *T. denitrificans* in the dilution used for isolation. All the other isolates grew heterotrophically on AH medium and consequently were not of the genus *Thiobacillus*. However, they may have belonged to the facultatively autotrophic thiobacillus-like bacteria described by Tuttle & Jannasch (1972), who stated that biological oxidation of reduced sulphur compounds was mainly performed by facultatively autotrophic bacteria. Tuttle *et al.* (1974) confirmed the occurrence of such organisms and described three isolated strains of marine-heterotrophic bacteria able to denitrify and oxidize organic compounds and thiosulphate simultaneously. Unfortunately, our heterotrophically growing strains although isolated from plates with TD medium, were not retested for their ability to utilize thiosulphate as hydrogen donor.

Table 6.6. Counts of thiobacillus-like bacteria per g of wet sediment under field conditions (before incubation) and after anaerobic incubation with nitrate at 15°C under laboratory conditions. During incubation, hydrogen donors from the sediment were utilized. Colonies were counted on TD medium under anaerobic conditions in the presence or absence of nitrate.

Time of counting	Counts on plates	
	with NO_3^-	without NO_3^-
Before incubation	1.37×10^6	$< 0.01 \times 10^6$
After incubation	960×10^6	$< 0.01 \times 10^6$

Table 6.7. Utilization of several organic compounds by heterotrophic denitrifying strains.

Genus	Number of strains examined	Number of strains utilizing											
		Cellulose	Xylan	Pectin	Cellobiose	Gal UA[a]	Xylose	Arabinose	Galactose	Mannose	Glucose	Na-lactate	Na-acetate
Pseudomonas	7	0	6	1	7	3	0	4	5	6	7	7	7
Alcaligenes	4	0	1	0	3	1	1	1	3	2	4	4	4
Micrococcus	1	0	1	0	1	0	1	0	1	1	1	1	1
Spirillum	1	0	0	0	0	0	0	1	0	1	1	1	1

[a] Galacturonic acid.

Growth of heterotrophic denitrifying isolates on hydrogen donors

Growth was tested of some representatives of the denitrifying population isolated from sediment after anaerobic incubation with nitrate (Table 6.5): 7 isolates of *Pseudomonas*, 4 isolates of *Alcaligenes*, the one of *Micrococcus* and the one of *Spirillum*. These 13 strains were tested for growth on various organic compounds likely to occur in the sediment: cellulose, cellobiose, xylan, pectin, galacturonic acid, galactose, mannose, glucose, xylose, arabinose, sodium acetate and sodium lactate (Table 6.7). All these compounds except cellulose were utilized by at least one (pectin) but mostly more than one representative of the denitrifying population as occurring in the sediment after anaerobic incubation with nitrate (Table 6.5). These isolates were present in counts of at least 10^6 in 1 g wet sediment (Table 6.3). Consequently the denitrifying population of the sediment must have been able to utilize components of the sediment as listed in Table 6.7, except cellulose.

Attempts to isolate bacteria simultaneously utilizing methane and nitrate under anaerobic conditions, failed. This result is in disagreement with the report of Davis (1973), who stated that such organisms may easily be isolated from activated sludge.

Oxidation of thiosulphate to tetrathionate by heterotrophic denitrifying bacteria

The above-mentioned 13 isolates were tested for their capacity to oxidize thiosulphate to tetrathionate. Two strains of the genus *Pseudomonas*, one of *Alcaligenes* and the *Spirillum* strain were able to carry out this oxidation. The contribution of these bacteria, with counts of at least 10^6 in 1 g wet sediment to the oxidation of sulphide in anaerobic sediments in the presence of nitrate is still unknown.

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7 Availability of hydrogen donors for denitrifying bacteria in aquatic sediments

Introduction

Removal of nitrate from natural waters results from denitrification in anaerobic aquatic sediments (Chapters 2 and 3). During this process, denitrifying bacteria utilize organic and possibly inorganic compounds as hydrogen donor of the sediment. In natural waters, insoluble organic material (like residues of higher plants and phytoplankton) is continuously precipitated from the overlying water to the sediment. Knowledge of the chemical composition of this sedimentary material can give information about its availability as hydrogen donors for denitrifying bacteria.

Only a few papers dealing with the chemical composition of organic matter in sediments can be found in present-day literature. Kemp & Mudrochova (1973) found that less than 0.25% of the total nitrogen in Ontario Lake sediments consisted of free amino acids, soluble combined amino acids and amino sugars, 49–55% of insoluble combined amino acids and amino sugars, and about 38–44% of unknown organic nitrogenous compounds.

The concentration of free sucrose, glucose, galactose, fructose, arabinose, xylose and ribose in aquatic sediments of Connecticut ranged from 13 to 191 μg per g dry sediment for each sugar mentioned (Vallentyne & Bidwell, 1956). In sediments of three Ontario lakes the total amounts of free maltose, sucrose, glucose, galactose, fructose, arabinose, xylose, ribose and two unknowns ranged from traces to 2.9 mg per g of ignitable matter (Whittaker & Vallentyne, 1957). Acid hydrolysis of near-surface bottom sediments from lakes of central Minnesota gave rise to eight sugars: arabinose, xylose, galactose, glucuronic acid, glucose, rhamnose, mannose and ribose. The concentration of each compound ranged 0.1–19.1 mg per g of dry sediment (Rogers, 1965).

Some more information about the chemical composition of organic matter of aquatic sediments can be obtained from geochemical studies by Rittenberg *et al.* (1963) and Swain (1966).

In the present study, firstly the amino acid and carbohydrate patterns were determined in aqueous extracts and in acid hydrolysates of two types of sediment. Secondly, a few experiments are described that showed the availability of some inorganic and organic compounds as hydrogen donor for aerobic, anaerobic, and denitrifying bacteria in sediments.

Materials and methods

Sediments

Two widely differing types of sediment were used, of which one type (A) originated from the upper 10-cm bottom layer of a ditch receiving effluent from a waste water

purification plant for slurry from calves, destined for meat production, and the other type (B) from the upper 10-cm bottom layer of a ditch receiving drainage water of arable land. The sediments were of the same origin as in previous investigations.

Before use, the wet sediment was graded with a sieve of mesh 2 mm to remove stones and subsequently the wet sediment was homogenized.

Amino acid composition

To estimate the 'free' amino acids of sediment, about 10 g wet sediment was mixed vigorously with 50 ml glass-distilled water for 30 minutes. The slurry was centrifuged and subsequently the supernatant was sucked off. This procedure was repeated twice. The collected supernatants were desalted with the cation-exchange resin Amberlite IR–120 (H^+). The amino acids were eluted from the resin with 0.5 N NH_3 and the eluate was evaporated under vacuum at 40°C to dryness with a rotary evaporator. The residue was dissolved in 5 ml buffer solution of pH 2.2 (Van Egeraat, 1972) and analysed for amino acids.

To estimate total amino acids about 4 g dry sediment was mixed with 20 ml 6 N HCl. Hydrolysis was carried out in a sealed glass tube for 16 hours at 110°C. The hydrolysed sample was neutralized with concentrated NaOH. A clear hydrolysate was recovered by centrifuging, whereafter the hydrolysate was desalted and treated further as in the previous paragraph.

Combined amino acids were calculated by subtracting values for free amino acid from those for total amino acids.

Distribution of carbohydrates

Fresh wet sediment was dried and homogenized by ball-milling. The distribution of carbohydrates in the sediment was derived from the contents of hexoses, pentoses and hexuronic acids in four carbohydrate extracts. These extracts were obtained by treating sediment in the following 4 ways:

Extract 1. To determine water-soluble carbohydrates, a mixture of 10 g of dry sediment and 50 ml distilled water was stirred vigorously for 15 min. Then the suspension was centrifuged. After filtering the supernatant through a glass fibre paper (Whatman GF/C), the clear solution was used for carbohydrate analysis.

Extract 2. Into a glass tube were introduced 10 g dry sediment and 50 ml distilled water. After sealing the tube, it was heated at 105°C for 16 hours. During this heating process, the tube was shaken a few times. After cooling, the suspension was centrifuged. The supernatant was filtered through a glass fibre paper.

Extract 3. Into a glass tube were introduced 3 g dry sediment and 50 ml 2 N H_2SO_4. The tube was sealed and heated for 16 hours at 100°C. During hydrolysis, the tube was shaken a few times. After cooling, the slurry was centrifuged. The supernatant was filtered through a glass fibre paper after which the solution was used for carbohydrate analysis.

Extract 4. To solubilize the cellulosic carbohydrates, a mixture of 3 g dry sediment and 4 ml of 72% H_2SO_4 was prepared in a glass tube and kept at room temperature for 4 hours. Subsequently, 50 ml distilled water was added, after which the tube was sealed and

the contents hydrolysed completely at 100°C for 6 hours, the tube being shaken a few times. After cooling, the slurry was centrifuged. The supernatant, after filtering through a glass fibre paper, was used for carbohydrate analysis.

Carbohydrate groups were estimated colorimetrically without further treatment of the extracts and by gas-liquid chromatography with Extracts 3 and 4, which were neutralized by adding solid $Ba(OH)_2$. Precipitated $BaSO_4$ was removed by centrifuging.

All extracts were deep-frozen until use.

Components of the sediment used as hydrogen donor in denitrification

Experiment 1. Contribution of lower fatty acids to denitrification in sediments

Two tubes (I.D. 3.4 cm), containing about 100 g wet sediment of type A, were supplied with 20 ml neutral solution containing 10 mg $NaNO_3$-N (1 tube) or with 20 ml water. The sediment surface was not disturbed during addition of the liquid. Then the tubes were incubated anaerobically at 25°C in the dark in an anaerobic jar (Chapter 6). The concentrations of nitrate and the lower fatty acids C_2–C_5 in the overlying water of both tubes were determined several times during incubation. If necessary, the amount of nitrate nitrogen in the overlying water in the tube with nitrate was completed to 10 mg. The presence of low-molecular alcohols in the overlying water of both tubes was tested once.

Experiment 2. Contribution of sulphide to denitrification in sediments

Twenty-one culture tubes were each filled with 2 g wet sediment of type A. To each of 14 tubes was carefully added 10 ml of a solution containing 6 mg $NaNO_3$-N and to each of the other tubes 10 ml water. All tubes were incubated anaerobically at 25°C in the dark in an anaerobic jar. The concentrations of nitrate, ferric iron and sulphate were estimated several times during incubation. For that purpose, three tubes, two with and one without nitrate, were taken from the jar at each date of analysis. One tube with nitrate was used for estimating nitrate. The two other tubes were directly acidified with 1 ml 0.5 N HCl, stirred and the contents subsequently filtered. The entire filtrates were used for estimating ferric iron and sulphate. At the 14th day of incubation the remaining nitrate tubes were supplemented with 6 mg $NaNO_3$-N per tube.

Experiment 3. Contribution of carbohydrates to denitrification in sediments

Three tubes (I.D. 3.4 cm) received each 7 g of wet sediment A, and 3 other tubes, 15 g of wet sediment B. At the same time, large portions of the sediments were dried, subsequently ball-milled and stored for chemical analysis. One hundred ml of a solution containing 100 mg l^{-1} $NaNO_3$-N was carefully added to one tube and 100 ml water to each of both other tubes. One of the tubes without added nitrate was incubated aerobically. This tube was weighed at regular intervals for measuring evaporation losses during the experiment. The two other tubes were sealed with a rubber stopper, provided with a small glass tube (Fig. 4.1) using a permanently elastic paste based on butyl-rubber to prevent leakages. For 10 minutes, nitrogen was bubbled through the water and the air

space of the tubes to remove oxygen. Immediately thereafter, Terumo caps were placed on the small glass tube. All of the sediment-containing tubes were incubated at 25°C in a dark room. At several times during incubation, H_2, CH_4 and CO_2 were measured in the gas phase of the closed tubes, NO_3^- was determined in the overlying water of the closed tube supplied with nitrate, and lower fatty acids were measured in the overlying water of all tubes. For that purpose, small gas and water samples were taken from the closed tubes through the Terumo cap with a syringe. The approximate pH of the samples of the overlying water was measured with pH indicator paper strips. Any possible gas leakage caused by small holes left in the cap by the needle was prevented by smearing the top of the cap with a thin layer of high-vacuum grease. Incubation was stopped when the nitrate consumption in the closed tube with nitrate approached zero. Then, the contents of the tubes were dried, weighed and subsequently ball-milled. Two portions of 2.3 g each of dry sediment A and two portions of 5 g of sediment B derived from each tube were hydrolysed by the procedure for Extract 4. The hydrolysates were used for carbohydrate analysis using the GLC technique. The remainder of each sediment sample was used for estimating hexuronic acids.

The present experiment was carried out in duplicate and at least repeated once with both types of sediment.

Amino-acid analysis

These analyses were with a Biocal-200 amino-acid analyser, by the procedure of Moore & Stein (1954) as described by Van Egeraat (1972).

Carbohydrate analysis

Carbohydrate groups were estimated colorimetrically with dry ball-milled sediment samples and with hydrolysates of sediments. Hexoses were determined by the anthrone reaction (Koehler, 1952), pentoses by the orcinol reaction (Bial, 1962) and hexuronic acids with the carbazole-sulphuric acid reagent (Bitter & Muir, 1962). The carbohydrate values obtained by these colorimetric methods were corrected for the interaction of concentrated acid with non-carbohydrate organic constituents in sedimentary samples and hydrolysates, because this interaction caused interfering absorbance. The orcinol reaction could not be carried out with sediment samples because of a very great interfering absorbance.

Gas-liquid chromatography (GLC) was used for separating individual hexoses and pentoses in hydrolysates as alditol-acetate derivatives. The conversion of hexoses and pentoses into these derivatives was performed according to Zevenhuizen (1973). To quantify this method, first meso-inositol and 2-deoxy-D-glucose were used as internal standards (Sloneker, 1971). Although Sloneker obtained good results with hydrolysates of plant tissues, the method did not work satisfactorily with hydrolysates of sediments. Therefore, later on the quantification was performed by separately measuring the glucose concentration in neutralized hydrolysates by the specific reaction with D-glucose-oxidase (AB KABI, Stockholm) according to Fales (1963). Sugars were identified by comparing retention times of unknown peaks in the GLC with those of known reference sugars.

Gas-chromatographic analysis

In the gas phase, H_2, CH_4 and CO_2 were determined by using a Becker type 406 gas chromatograph equipped with a thermal conductivity detector with W2X filaments and a filament current of 100 mA. A stainless steel column (300 cm × 2 mm) was used containing Porapak Q (80–100 mesh) at 50°C with molecular nitrogen as carrier gas (flow 25 ml min^{-1}).

Lower fatty acids (C_2–C_5) were analysed by a method described by Van der Laan (1974). A Becker type 409 gas chromatograph was used, equipped with a flame-ionization detector and a glass column (100 cm × 4 mm) containing 20% Tween 80 on Chromosorb W-AW (80–100 mesh). Nitrogen saturated with formic acid was the carrier gas (flow 80 ml min^{-1}). The temperature settings were: injection port 170°C, column oven 115°C and detector 170°C.

Alditol acetates were separated by the method of Lönngren & Pilotti (1971) by using a Becker Unigraph-F type 407 gas chromatograph, equipped with a flame-ionization detector. A stainless steel column (200 cm × 4 mm) containing 3% of OV-225 on Chromosorb W-HP (100–120 mesh) was used, whilst nitrogen was the carrier gas (30 ml min^{-1}). The column temperature was 200 °C.

Low-molecular alcohols (C_1–C_4) were analysed by using a Becker Multigraph type 409 gas chromatograph equipped with a flame-ionization detector and a glass column (110 cm × 4 mm) containing Chromosorb 101. Nitrogen was used as carrier gas (flow 25 ml min^{-1}). The temperature settings were: injection port 165°C, column oven 155 °C and detector 165 °C.

Other chemical analyses

Nitrate was determined by the salicylate method described by Müller & Widemann (1955). Sulphate ion was determined by a turbidimetric method involving the production of an unstable suspension of $BaSO_4$ as described by Golterman (1970). Ferric iron was estimated with Tiron reagent as described by Beck (1959). Ammonium and organic nitrogen in the sediment were estimated by the method of Bremner (1965).

Results and discussion

Amino acid composition of aqueous extracts (free amino acids) and acid hydrolysates (free and combined amino acids) of sediments

Amino acids were determined in sediment samples A, A* and B. Sample A was taken from the ditch near the discharge point of effluent, sample A* about 600 m downstream from that point. The organic matter contents of these sediments were 37.4 (A), 28.9 (A*) and 14.2 (B) mg per g dry sediment, respectively. Near the discharge point, the sediment contained more nitrogenous organic matter than at a considerable distance downstream (Table 7.1). B contained less nitrogenous organic matter than A and A*. Contents of free amino acids in the three sediments were very low, but they were much higher than those found by Kemp & Mudrochova (1973, 1975) in Lake Ontario surface sediments. The total contents of nitrogen, occurring as combined amino acids in the three sediments,

amounted to 681, 80 and 12 μg N per g dry sediment corresponding to 41.5, 16.3 and 4.6%, respectively, of the total sediment nitrogen. The percentages of nitrogen occurring as combined amino acids in sediments A* and B were lower than those found by Kemp & Mudrochova (1973, 1975). Only the value of A corresponds with the values reported by them. The decreasing percentages of combined amino acids in the sediment range A, A* and B may be explained by the increasing age of the organic material in these sediments.

The amino acid patterns of the fractions of free and combined amino acids differed mutually as well as between sediments of different origin (Table 7.2). Aspartic acid,

Table 7.1. Nitrogen distribution in three aquatic sediment samples (μg N per g dry sediment).

Nitrogen occurring in	A	A*	B
Free amino acids	13 (0.8)[a]	6 (1.2)	1 (0.4)
Combined amino acids	681 (41.5)	80 (16.3)	12 (4.6)
Unknown organic compounds[b]	566 (34.5)	324 (66.1)	211 (81.2)
Free and fixed ammonia	380 (23.2)	80 (16.3)	30 (11.5)
Total	1640	490	260

[a] Figures in parentheses are nitrogen of each fraction as % of total nitrogen of each sample.
[b] Values obtained by subtracting estimated nitrogen from total nitrogen.

Table 7.2. The amino-acid composition of the fractions of free and combined amino acids. (Amino acids expressed as mole percent of each fraction)

	Free amino acids			Combined amino acids		
	A	A*	B	A	A*	B
Cysteic acid	tr	nd	nd	tr	1.4	1.5
Aspartic acid	8.7	13.6	2.5	8.2	23.0	15.1
Threonine	4.3	nd	tr	3.5	4.8	6.1
Serine	7.2	36.4	19.0	6.2	12.2	1.1
Glutamic acid	10.1	9.1	13.9	7.7	12.4	nd
Proline	2.9	nd	nd	5.7	4.4	5.4
Glycine	11.6	27.3	12.7	17.7	10.1	11.8
Alanine	10.1	nd	13.9	12.7	tr	17.3
Valine	4.3	4.5	13.9	5.5	3.9	9.0
Cystine	nd	nd	nd	nd	nd	nd
Methionine	5.8	nd	6.3	1.2	nd	1.5
Isoleucine	4.3	4.5	3.8	4.3	2.5	4.7
Leucine	7.2	4.5	5.1	7.2	3.5	5.7
Tyrosine	1.4	nd	tr	1.6	2.5	5.1
Phenylalanine	1.4	nd	tr	3.8	3.2	3.5
Tryptophan	1.4	nd	nd	2.1	nd	1.4
Lysine	11.6	nd	nd	10.0	5.8	8.7
Histidine	tr	nd	8.9	tr	3.9	0.6
Arginine	7.2	nd	nd	2.7	6.2	1.4

tr = trace, nd = not detectable.

serine, glutamic acid, glycine, alanine and valine were the predominant free amino acids, aspartic acid, glycine and alanine were the predominant combined amino acids. These amino acids were also found in the highest concentrations in humic acid from soil (Huntjens, 1972) and in humic and fulvic acid fractions from sediments of Lake Ontario (Kemp & Mudrochova, 1975). This indicates that part of the amino acids in the sediments tested was present in some sort of association with humic substances.

Distribution of carbohydrates

Some chemical data for the samples of sediments A and B used in this section are presented in Table 7.3. The organic matter content in sediment A was 3 times as high as that of B.

Total amounts of hexoses, pentoses and uronic acids were determined in the four extracts of each sediment sample (Table 7.4). Water-soluble monosaccharides and disaccharides dissolved in Extract 1. In Extract 2, some polysaccharides like starch and fructosan dissolved as well as the carbohydrates of Extract 1. Products of hydrolysis of hemicellulose and other easily hydrolysable water-insoluble polysaccharides as well as to the carbohydrates of Extract 2 dissolved in Extract 3. Cellulose and other polysaccharides resistant to treatment with 2 N H_2SO_4 hydrolysed as well as the carbohydrates of Extract 3 during the preparation of Extract 4. It was assumed that the carbohydrates of Extracts 3 and 4 were completely hydrolysed and therefore were present as monosaccharides.

Less than 6% of the total carbohydrates of sediments A and B was present as water-soluble sugars (Table 7.4, Extract 1). In this extract, the following monosaccharides were detected: rhamnose/fucose, arabinose, xylose, mannose, galactose, glucose and 3 unknowns. More carbohydrates dissolved in boiling water (Extract 2); a large amount of pentoses was found with sediment A. Starch did not seem to be an important sugar component in either sediment as the hexose contents in Extract 2 remained relatively small. A large part of the carbohydrates of both sediments dissolved by treatment with 2 N H_2SO_4 at 100°C. This indicates that roughly 55% of the carbohydrates of both sediments consisted of hemicelluloses and other easily hydrolysable water-insoluble polysaccharides. Treatment with 72% H_2SO_4 resulted in an increase in the amounts of hexoses and to a less extent uronic acids, while that of pentoses slightly diminished (Extract 4) probably due to destruction of part of the pentoses (Rhiem, 1962). The qualitative carbohydrate analysis of Extracts 3 and 4 of both sediments, using the GLC

Table 7.3. Some chemical data (mg per g dry weight) of the samples of sediments A and B used for the determination of the distribution of carbohydrates.

	Sediment	
	A	B
Organic matter determined as volatile solids	59.6	19.6
Ammonium nitrogen	0.07	0.02
Organic nitrogen	0.98	0.22
pH	8.50	7.55

Table 7.4. Total contents of hexoses, pentoses and uronic acids as determined in the four collected extracts of sediments A and B.

Extract	Hexoses[a]	Pentoses[b]	Uronic acids[c]	Total	
				μg	%
Sediment A					
1: cold water	120	20	80	220	5
2: boiling water	330	730	230	1290	30
3: 2 N H_2SO_4	1160	1150	1200	3510	83
4:72% H_2SO_4	1840	960	1240	4230[d]	100
Sediment B					
1: cold water	30	20	20	70	3
2: boiling water	190	70	230	490	19
3: 2 N H_2SO_4	540	1060	500	2100	80
4: 72% H_2SO_4	940	810	640	2640[d]	100

[a] Expressed as μg glucose per g dry sediment.
[b] Expressed as μg xylose per g dry sediment.
[c] Expressed as μg glucuronic acid per g dry sediment.
[d] This value was obtained by adding up hexoses and uronic acids of Extract 4 and pentoses of Extract 3.

technique, showed that the increase of hexoses in Extract 4 of both sediments was caused by glucose and to a less extent by mannose. The large increase of glucose upon treatment with 72% H_2SO_4 indicates that cellulose is an important component of carbohydrates in both sediments.

One gram of the dry sample of sediment A contained 4.2 mg carbohydrate, which consisted of 43% hexoses, 27% pentoses and 29% uronic acids. The 2.6 mg carbohydrates found in one gram of a sample of sediment B was made up of 36% hexoses, 40% pentoses and 24% uronic acids. In sediments A and B, 7 and 13% of the organic matter, respectively, consisted of carbohydrates (Tables 7.3 and 7.4). Considering also free and combined amino acids, it is obvious that in both sediments most of the organic matter consisted of other compounds like lignic and humic substances.

Components of the sediment used as hydrogen donor in denitrification

Hydrogen donors are contained in the precipitated residues of higher plants, animals and phytoplankton or derived from these residues by anaerobic microbial processes. Without oxygen or nitrate in the overlying water, the soluble compounds diffuse into the overlying water, where they can be estimated. In the presence of oxygen and nitrate in the sediment, these compounds would presumably be oxidized. Three experiments were designed to demonstrate the role of lower fatty acids, sulphide and carbohydrates as hydrogen donors for aerobic, anaerobic, and denitrifying bacteria.

Experiment 1. Contribution of lower fatty acids to denitrification in sediments

This experiment was performed to measure the accumulation of lower fatty acids and alcohols in the overlying water of an anaerobic water-sediment system with and without nitrate added.

The content of organic matter in the sample of sediment A amounted to 30 mg per g dry sediment. The thickness of the layer of sediment in both tubes was about 7 cm.

The consumption of nitrate by denitrifying bacteria in the sediment is presented cumulatively in Figure 7.1. During incubation, the upper 2 cm layer of sediment in the tube with nitrate in the overlying water gradually turned greyish brown. The underlying sediment and the entire sediment layer in the tube without nitrate maintained their black colour. Lower fatty acids were not detected in the overlying water of the tube with added nitrate, but they accumulated in the overlying water of the tube without nitrate (Fig. 7.2). Of low-molecular alcohols in the overlying water of both tubes, a trace of ethanol was present only in the tube without nitrate. From the results, it is evident that during fermentation of the organic matter present in the sediment, mainly acetic acid is formed. About 90% of the lower fatty acids was this acid whilst the remaining lower fatty acids consisted of propionic, isobutyric, butyric, isovaleric and valeric acids.

The thickness of the greyish brown upper layer of sediment indicates the depth of nitrate penetration into the sediment (Chapters 4 and 5). Consequently, nitrate would have been contained only in the upper 2-cm layer of the sediment in the tube with nitrate added, so that the conditions in the lower 5-cm layer of the sediment would be identical with those of the entire sediment of the tube without nitrate. Therefore, acetic acid was undoubtedly formed in large amounts in the tube with nitrate, even though no production of this acid would have occurred in the upper layer. As no acetic acid was detected in the overlying water, it is obvious that after upward diffusion into the nitrate-containing zone, this acid was utilized by denitrifying bacteria as hydrogen donor according to

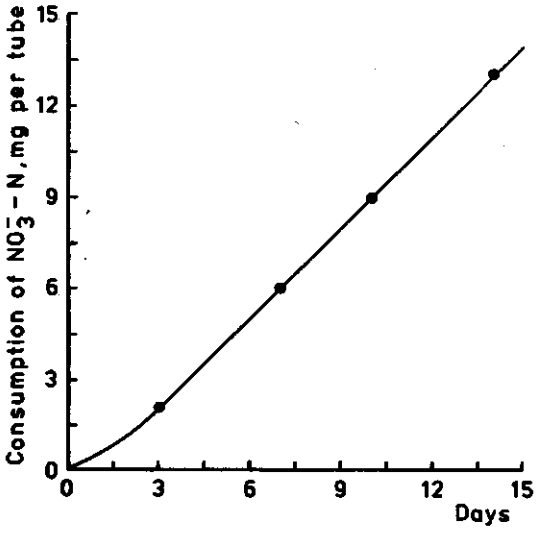

Fig. 7.1. Nitrate nitrogen consumed by the sediment of Experiment 1 during anaerobic incubation at 25°C.

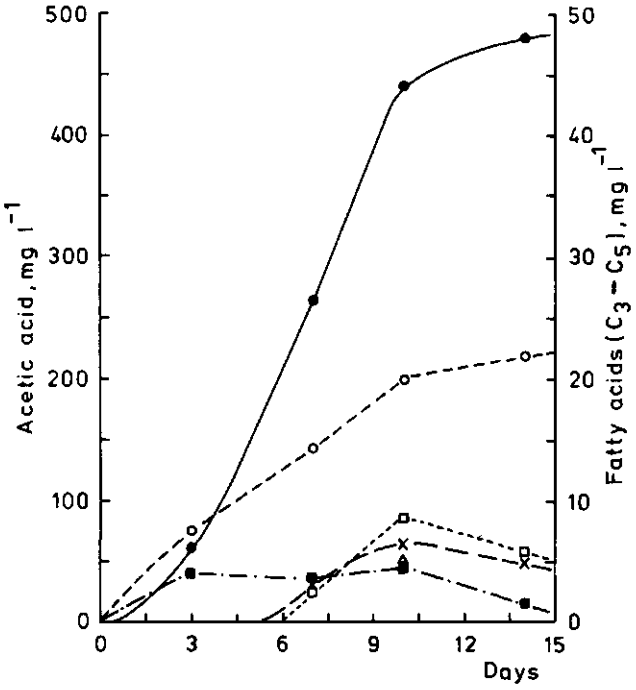

Fig. 7.2. Accumulation of lower fatty acids in the overlying water of sediment incubated anaerobically at 25°C in the absence of nitrate.
●———● Acetic acid; ○– – –○ Propionic acid; X– –X Isobutyric acid; ■–·–·–■ Butyric acid; □·····□ Isovaleric acid; △ Valeric acid.

$$5\ CH_3COOH + 8\ NO_3^- \rightarrow 4\ N_2 + 10\ CO_2 + 8\ OH^- + 6\ H_2O \qquad (1)$$

From this reaction the amount of NO_3^- required for the complete oxidation of the acetic acid (derived from the anaerobic tube without NO_3^-, Fig. 7.2) available for denitrification can be calculated. Actually, this amount has to be corrected for an unknown amount of acetic acid used for cell synthesis of the denitrifying bacteria. The calculated amount of nitrate used for the oxidation of acetic acid and the total amount of nitrate consumed in this tube (Fig. 7.1) suggest that about 30% of the disappeared nitrate was consumed by denitrifying bacteria utilizing acetic acid as hydrogen donor.

Experiment 2. Contribution of sulphide to denitrification in sediments

The fading of the upper layer of black sediment during anaerobic incubation in the presence of nitrate was ascribed earlier (Chapters 4 and 5) to the oxidation of FeS. The aim of this experiment was to demonstrate the formation of sulphate and ferric iron, causing the fading under such conditions.

Some chemical data on the sample of sediment A used in this experiment are presented in Table 7.5. The amount of nitrate consumed by the denitrifying bacteria in 2 g of wet sediment during anaerobic incubation at 25°C is presented cumulatively in

Table 7.5. Some chemical data (mg per g of dry sediment) of the sample of sediment A used in Experiment 2.

Water	575
Organic matter determined as volatile solids	134.1
Ammonium nitrogen	0.37
Organic nitrogen	4.20
pH	7.40

Figure 7.3. During this consumption, the sediment turned greyish brown, whereas in the absence of nitrate it remained black. After the 18th day of incubation, practically no more nitrate was consumed, probably through lack of hydrogen donors. It should be noted that the amount of sediment used in this experiment was much smaller than in Experiment 1.

The amount of sulphate in the overlying water of the nitrate-treated tubes increased during the first 18 days of incubation (Fig. 7.4). Afterwards, sulphate formation and nitrate consumption had practically come to a standstill. The small amounts of sulphate detected in the overlying water under anaerobic conditions without added nitrate were presumably formed chemically during the sulphate determination.

The oxidation of sulphide to sulphate under anaerobic conditions in the presence of nitrate can be carried out by two types of sulphide-oxidizing bacteria: nitrate-reducing and denitrifying bacteria according to the respective reactions

$$S^{2-} + 4\,NO_3^- \rightarrow SO_4^{2-} + 4\,NO_2^- \qquad (2)$$

$$5\,S^{2-} + 8\,NO_3^- + 4\,H_2O \rightarrow 5\,SO_4^{2-} + 4\,N_2 + 8\,OH^- \qquad (3)$$

The formation of sulphate by the former type of bacteria is most likely, as only such organisms were isolated from the sediment (Chapter 6). Nitrite did not accumulate,

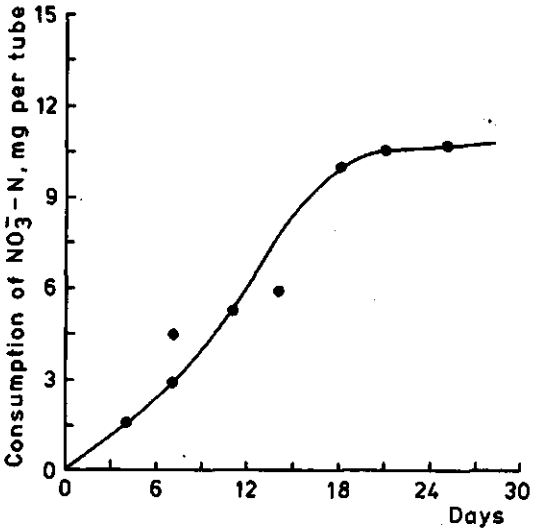

Fig. 7.3. Nitrate nitrogen consumed by the sediment of Experiment 2 during anaerobic incubation at 25°C.

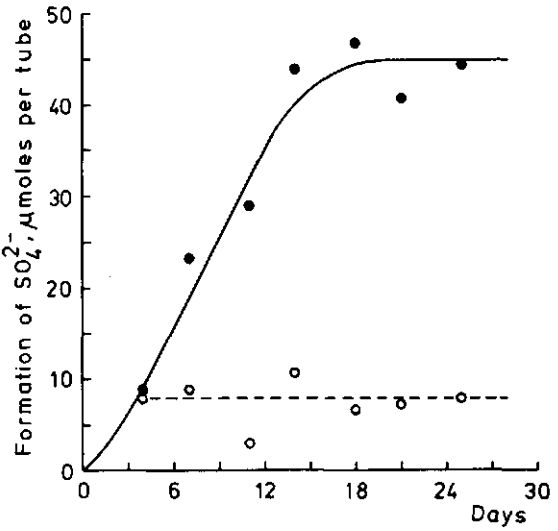

Fig. 7.4. Sulphate in the overlying water of a sediment incubated anaerobically at 25°C in the presence and absence of nitrate. ●——●——● With nitrate; o---o Without nitrate.

because it was easily further reduced to molecular nitrogen by denitrifying bacteria using organic compounds as hydrogen donor. From Figure 7.4 and Reaction 2, it can be calculated that about 2.5 mg NO_3^--N had to be reduced to NO_2^--N for the formation of the ultimate amount of sulphate present. This amount corresponds with 1 mg NO_3^--N being reduced to molecular nitrogen, which is equal to 10% of the disappeared nitrate (Fig. 7.3).

Experiment 3. Contribution of carbohydrates to denitrification in sediments

This experiment was carried out several times with both types of sediment. It failed often through difficulties with the carbohydrate analysis by GLC or colorimetry attributable to interference with unknown compounds, particularly in sediments of type A. In sediment B, these interfering compounds were present to a less extent, so that the availability of the carbohydrates for microorganisms could be studied.

Some chemical data on the sediment of type B are presented in Table 7.6. The carbohydrates of that sediment were measured as monosaccharides upon hydrolysis with 72% H_2SO_4 (Table 7.7). The monosaccharides originally occurred for more than 50% as

Table 7.6. Some chemical data (mg per g dry sediment) of the sample of sediment B used in Experiment 3.

Water	286
Organic matter determined as volatile solids	19.6
Ammonium nitrogen	0.02
Organic nitrogen	0.22
pH	7.55

Table 7.7. Glucose, galactose, mannose, xylose, arabinose, rhamnose/fucose and uronic acids (mg per tube) in tubes with sediment B before incubation, after anaerobic incubation with and without NO_3^- added, and after aerobic incubation without NO_3^- added.

	Before incubation	After 42 days of incubation		
		anaerobic		aerobic
		with NO_3^-	without NO_3^-	without NO_3^-
Glucose	4.4	1.3	1.9	0.7
Galactose	1.4	0.2	0.3	0.1
Mannose	0.6	0.7	0.3	0.3
Xylose	1.4	0.1	0.2	0.1
Arabinose	1.2	0.4	0.4	0.2
Rhamnose/fucose	0.4	0.1	0.2	0.1
Uronic acids[a]	1.5	1.3	1.8	1.2
Total	10.9	4.1	5.1	2.7

[a] Expressed as mg glucuronic acid per tube.

hemicellulosic compounds, as has been shown in section 'Distribution of carbohydrates'.

During the 42 days of incubation of the water-sediment system at 25°C, the pH of the overlying water in all tubes remained between 6.5 and 7.5. The black sediment turned entirely yellow-brown in the tubes with nitrate added, whereas in the absence of nitrate it kept black during incubation. The upper half of the sediment in tubes incubated aerobically also turned yellow-brown.

In the closed anaerobic tubes without nitrate added, carbohydrates decreased by 5.8 mg when incubated for 42 days (Table 7.7). It is likely that the largest part of the disappeared carbohydrates had been fermented by facultatively and obligately anaerobic bacteria. During this fermentation, acetic acid was formed mainly, while the formation of propionic, isobutyric, butyric, isovaleric and valeric acid was insignificant (Fig. 7.2). Therefore, only the contents of acetic acid are presented in Figure 7.5. It can be seen that in the anaerobic tubes without nitrate added, acetic acid is gradually converted into methane.

In the anaerobically incubated tubes supplied with nitrate, carbohydrates decreased by 6.8 mg during incubation, but acetic acid and methane were not detected (Fig. 7.5). Hydrogen was formed under these conditions, but the amounts were small compared with those of CH_4 in the tubes without NO_3^-. As reported in Chapter 6, none of the denitrifying bacteria isolated from such tubes decomposed cellulose, though cellulose was an important component of the carbohydrate fraction in sediments (see section 'Distribution of carbohydrates'). The observation (Zaal, personal communication) that most of the facultatively and obligately anaerobic bacteria (including anaerobic cellulose-decomposers) isolated from sediment were able to ferment organic substrates in the presence of nitrate suggests that in the tubes supplied with nitrate part of the carbohydrates was transformed into acetic acid by anaerobic bacteria before being utilized as hydrogen donor by denitrifying bacteria according to Reaction 1. The remaining part of the disappeared carbohydrates was presumably directly utilized by denitrifying bacteria.

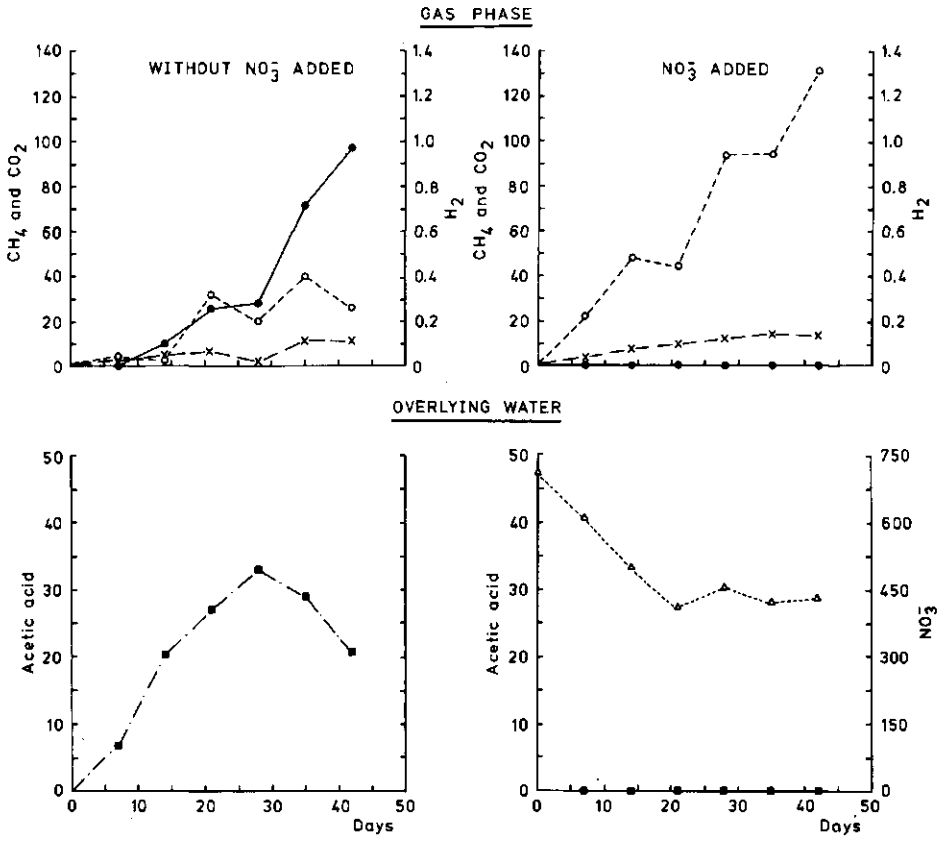

Fig. 7.5. H_2, CH_4 and CO_2 *in the gas phase and nitrate and acetic acid in the overlying water* (*μmoles* per tube) of two anaerobic water-sediment systems with and without added nitrate. ●——● CH_4; ○ - - - - - ○ H_2; × — —× CO_2; △.....△ Nitrate; ■—.—■ Acetic acid.

Hexoses and pentoses, for instance, can be converted anaerobically by denitrifying bacteria according to the respective reactions

$$5\ C_6H_{12}O_6 + 24\ NO_3^- \rightarrow 12\ N_2 + 30\ CO_2 + 24\ OH^- + 18\ H_2O \quad (4)$$

$$C_5H_{10}O_5 + 4\ NO_3^- \rightarrow 2\ N_2 + 5\ CO_2 + 4\ OH^- + 3\ H_2O \quad (5)$$

From Reactions 1, 4 and 5, one can calculate that 0.37 mg NO_3^--N is reduced to molecular nitrogen during conversion of 1 mg acetic acid or carbohydrate. Consequently, the disappeared carbohydrates (6.8 mg) have been respired by denitrifying bacteria reducing 2.5 mg NO_3^--N to molecular nitrogen, so that loss of carbohydrates accounted for 60% of the total consumption of nitrate. This value may be 10–20% lower as part of the carbohydrates has been used for cell synthesis of the denitrifying organisms.

From the aerobically incubated tube without nitrate, 8.2 mg carbohydrates disappeared. It is likely that oxygen was present only in the yellow-brown sediment layer (Chapter 5). In the anaerobic underlying sediment layer, a similar situation may be

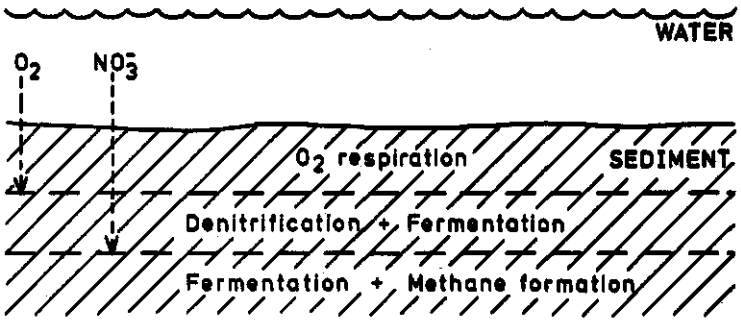

Fig. 7.6. Occurrence of three zones with different microbial activity in a sediment with overlying water containing nitrate as well as oxygen.

expected to have occurred as was found in the sediment of the anaerobically incubated tube without nitrate. All biologically degradable organic compounds of the aerobic layer and also compounds diffusing from the anaerobic layer into the aerobic layer would presumably be consumed by aerobic bacteria, utilizing oxygen as hydrogen acceptor. Lower fatty acids and methane belong to the latter group of compounds. This explains why lower fatty acids were not detected in the overlying water of the sediment.

Under natural conditions with nitrate and oxygen present in the overlying water of a water-sediment system, both hydrogen acceptors diffuse into the sediment (Chapter 5). As denitrification is strongly inhibited by oxygen, this process proceeds in the sediment below the layer where oxygen is consumed. In the denitrification zone of the sediment fermentation of organic material may proceed to some extent. In the layer below that zone, fermentation reactions and formation of methane occur (Fig. 7.6). As part of the metabolites derived from the fermentation processes in the lowest zone diffuse into the denitrification layer, denitrifying bacteria are in a privileged position compared with the oxygen-respiring bacteria for supply of hydrogen donors from deeper layers of the sediment.

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8 Removal of nitrate from effluent following discharge on surface water

Abstract

The loss of nitrate nitrogen over a 800-m long reach of a canal was studied in a field experiment during a 20-days period by analysing daily water samples from two sample stations, situated at the beginning and the end of the canal reach, respectively. The nitrate in the canal water originated mainly from effluent of a waste water purification plant. Fifty-six per cent of the nitrate present in the canal water at the beginning of the 800-m long reach had disappeared during its flow through the 800-m long reach. The average retention time of the canal water in the 800-m long reach was 1.7 days. The average rate of nitrate disappearance during the 20-days period was 537 mg NO_3^--N m^{-2} day^{-1}. Laboratory experiments with undisturbed water-sediment profiles from the canal showed that the above-mentioned disappearance of nitrate was caused mainly by denitrification in the sediment. Increased knowledge of this phenomenon may lead to an effective and cheap means in combating nitrogen-induced eutrophication problems in practice. These situations will occur as a result of non-point discharges of drain water from agricultural areas as well as by effluent discharges of waste water purification plants.

9A A simple respirometer for measuring oxygen and nitrate consumption in bacterial cultures

Abstract

A simple respirometer is described to measure simultaneously oxygen and nitrate concentrations. It proved to be an easy tool in denitrification studies. This was tested with *Pseudomonas aeruginosa*.

9B Respirometer experiments. Simultaneous measurements of the oxygen and nitrate uptake by aquatic sediments

Introduction

In a natural water-sediment system with nitrate and oxygen in the overlying water, nitrate was consumed by the sediment as shown by chemical analysis (Chapter 2). Nitrate was reduced mainly to molecular nitrogen (Chapter 3). Oxygen diffused from the overlying water into the sediment (Chapter 5). Edberg & Hofsten (1973), among others, measured the uptake of oxygen by sediments *in situ* and in the laboratory by using an oxygen-membrane electrode. They obtained rates in the range of 0.3–3.0 g O_2 $m^{-2}day^{-1}$ and observed that oxygen uptake by sediment in the laboratory gave consistently lower values than measurements *in situ*. In general, oxygen consumption by sediments is considered to be dependent on oxygen concentration in the overlying water. Edwards & Rolley (1965), Bouldin (1968), Fillos & Molof (1972) and Edberg & Hofsten (1973) developed various equations to describe this relationship. Hargrave (1972) showed that oxygen consumption was partly due to chemical oxidation in the sediment.

The aim of this study was simultaneously to measure uptake of oxygen and nitrate by a sediment core and by completely mixed sediment suspensions, using oxygen and nitrate electrodes.

Methods

The uptake of oxygen and nitrate by undisturbed sediment cores was measured with oxygen and nitrate electrodes. The device was a modification of the respirometer described in Chapter 9A (Fig. 9B.1). A polymethyl methacrylate tube (I.D. 6.2 cm; length 25 cm) was used to take water-sediment cores from shallow waters. A Jenkin mud sampler was used to take cores from deeper waters. The core was quickly transported to the laboratory with as little disturbance as possible. Thereafter, the tube with the core was immediately placed into a waterbath with a constant temperature equal to that of the core. N-Serve was added to the overlying water to a final concentration of 5 mg l^{-1} to prevent nitrification.

The top of the tube was closed with a polymethyl methacrylate lid, in which a nitrate electrode, a reference electrode, an oxygen-membrane electrode, a small hole for a rubber stopper, and a stirring-rod were fixed. The lid was provided with two rubber 'O' rings, by which the lid could easily be moved down into the tube until all the air below the lid had escaped through the hole for the rubber stopper. Then the hole was closed. The electrodes, their sealing in the lid, and their calibration procedures have been described in Chapter 9A. If necessary, sodium nitrate was added to the overlying water through a syringe past the rubber stopper. The stirring-rod was used to homogenize the enclosed mass of water without swirling the sediment. The whole experiment was performed in the dark. The two signals from the oxygen-membrane electrode and the nitrate electrode were

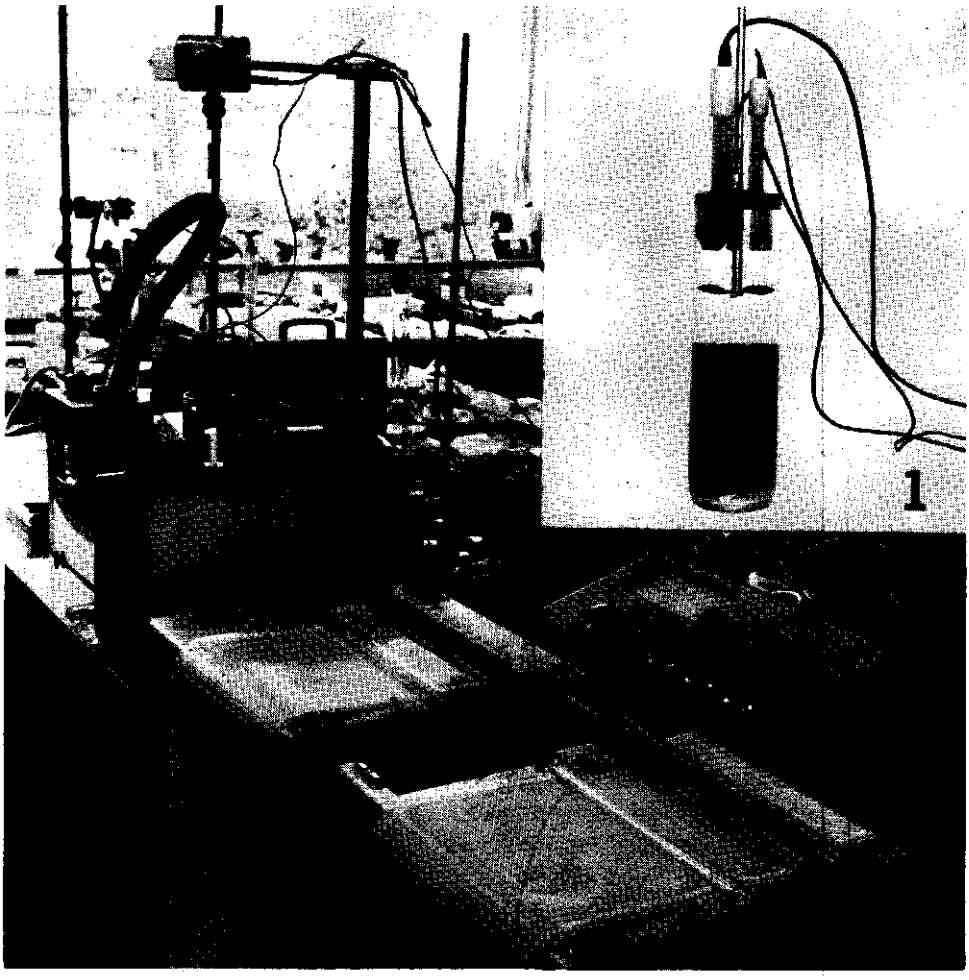

Fig. 9B.1. The measuring system for oxygen and nitrate uptake by undisturbed sediment cores.

registered by two recorders. From the enclosed mass of water and the area of the sediment surface, the uptake of oxygen and that of nitrate could be calculated and expressed in mmoles O_2 or NO_3^- m^{-2} day^{-1}.

The uptake of oxygen and that of nitrate at 25°C by suspensions of sediment were measured with the respirometer as described in Chapter 9A. Wet sediment 50 g was mixed vigorously with distilled water 100 ml and after precipitation for 10 sec, the suspension was poured into the respirometer. This precipitation left most of the grains of sand outside the respirometer, reducing wear on the respirometer.

Results and discussion

The uptake of oxygen and that of nitrate were measured with several undisturbed cores, which were taken from the ditch that contained sediment A (Chapter 2). The results of such a measurement are presented in Figure 9B.2. The volume of overlying

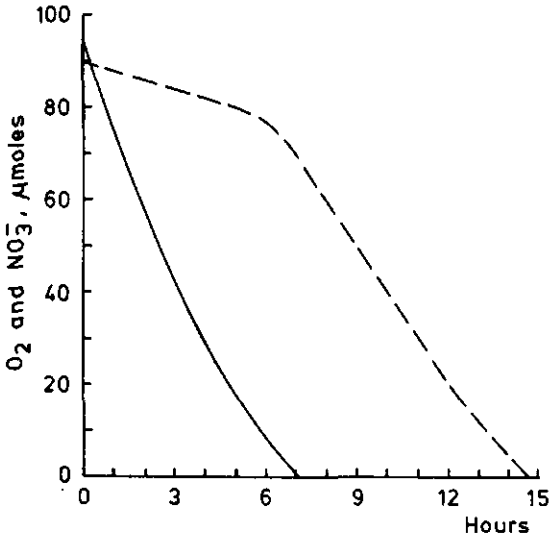

Fig. 9B.2. Amount of O_2 and NO_3^- (µmoles) in the overlying water of an undisturbed water-sediment core (area of sediment surface: 30 cm^2). ——O_2; – – – NO_3^-.

water was 370 ml. The core was kept at a temperature of 15°C. N-Serve was added to inhibit nitrification during measurement. In a preliminary experiment, the uptake rate of oxygen by overlying water was negligible compared with that by sediment. The average uptake rates of oxygen and nitrate by sediment amounted to 118.8 and 16.9 mmoles m^{-2} day^{-1}, respectively, during the first 6 h. The uptake rate of oxygen by sediment was much higher than that of nitrate when both hydrogen acceptors were present in the same substance concentration (expressed in µmoles l^{-1}) in the overlying water at the start of the experiment. After reaching a dissolved oxygen concentration of 1.5 mg l^{-1} (corresponding with 17 µmoles O_2 in Fig. 9B.2), the uptake rate of nitrate increased rapidly and after depletion of oxygen, it averaged 74.7 mmoles m^{-2} day^{-1}.

The adverse effect of oxygen in the overlying water on the uptake of nitrate by the sediment depends on the inhibitory effect of O_2 on denitrification. This effect increased with dissolved oxygen concentrations in the range from 0 to 1.5–2 mg l^{-1}. At higher concentrations of O_2, no further decreasing effect on denitrification (NO_3^- uptake) was found (Fig. 9B.2 and Fig. 2.7).

The denitrification rate found in the sediment core with oxygen present in the overlying water corresponded with that found in Chapter 2. However, the increase in rate of denitrification (Fig. 9B.2) was much more pronounced than in Chapter 2 (Fig. 2.7) when the concentration of O_2 fell from 1.5–2 mg l^{-1} to zero. No explanation can be given for this difference.

In another experiment, the presence of nitrate in the overlying water had no influence on the rate of oxygen uptake by the sediment. The ratio between the uptake rate of nitrate under anaerobic conditions and that of oxygen was 0.63.If the hydrogen donors were oxidized by nitrate and oxygen at equal rates, this ratio would be 0.80 (Reactions 1 and 2):

$$2\,NO_3^- + 10\,[H] \rightarrow N_2 + 2\,OH^- + 4\,H_2O \quad (1)$$
$$O_2 + 4\,[H] \rightarrow 2\,H_2O \quad (2)$$

The lower ratio found may indicate that (a) chemical oxidations proceed with O_2, (b)

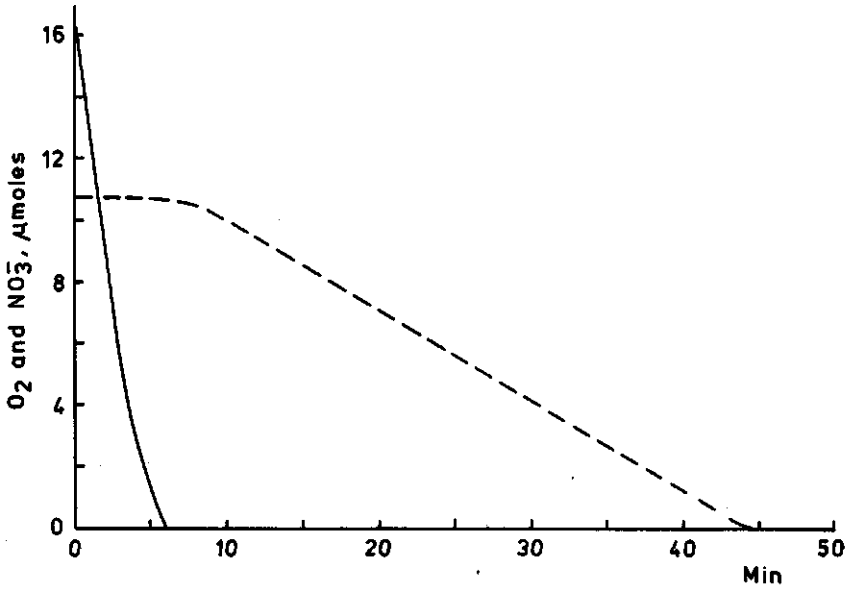

Fig. 9B.3. Amounts of O_2 and NO_3^- (µmoles) in a completely mixed suspension derived from 50 g wet sediment.
——O_2; – – – NO_3^-.

denitrifying bacteria utilize O_2 more readily than NO_3^-, (c) part of the aerobic bacteria is unable to denitrify.

Such respirometer measurements should have simplified some experiments with many time-consuming chemical analyses (Chapter 2). In practice, this expectation was not completely realized due to the complexity of the whole measuring device.

In completely mixed suspensions of sediment, uptake of nitrate started only after depletion of oxygen(Fig. 9B.3). This is in contrast with the results shown in Figure 9B.2, where nitrate uptake occurred, albeit at a reduced rate, in the presence of dissolved oxygen. In sediment suspensions, the ratio between the uptake rate of nitrate and oxygen was much smaller than that of the undisturbed sediment core. Sterilization of the suspension at 120°C or addition of cyanide resulted only in a slight reduction in uptake rate of oxygen, demonstrating that most of the oxygen taken up by the unsterilized suspensions was used for chemical oxidation of reduced compounds like sulphide. In the sterilized suspensions, uptake of nitrate was not measurable after oxygen depletion, so that nitrate was only consumed by nitrate-reducing and denitrifying bacteria.

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10 Summary and general discussion

Despite the increasing demand for natural waters of good quality, the dense population and many industries in the Netherlands produce increasing amounts of water-polluting matter. The need to prevent pollution of natural waters becomes increasingly urgent. Purification of waste water prevents deterioration of natural water only partly because inorganic nutrients, including nitrogen and phosphorus, mostly remain in the effluent in sufficiently large amounts to favour massive growth of algae (algal bloom), which spoils water quality. Drainage water from arable land may contain relatively large amounts of nitrate derived from fertilizer or from mineralized plant residues, washed out by excessive rain. The discharge of such water is also considered to contribute to eutrophication.

The aim of this study was to investigate the microbial transformations of nitrogenous compounds in natural waters, particularly those processes reducing the amount of available N. Of the various microbial processes occurring in natural waters, denitrification was thought to be most obvious, since nitrate, the final product of mineralization of organic N compounds, is converted to gases, which disappear into the atmosphere. As denitrification is an anaerobic process and natural waters mostly contain dissolved oxygen, the possibility of denitrification going on in the anaerobic sediment at the bottom of natural waters was considered.

Natural sediment with overlying water was used as a model system for studying denitrification in laboratory experiments. Two widely differing types of sediment were used, one (sediment A) originating from the upper 10-cm bottom layer of a ditch receiving effluent from a waste water purification plant for slurry from calves, and the second (sediment B) from a ditch receiving drainage water from arable land.

Chapter 2 reports the results of experiments on ammonification, nitrification and denitrification in water-sediment systems with sediment A at three incubation temperatures (4, 15 and 25°C). Inhibition of nitrification by N-Serve resulted in accumulation of ammonium in the overlying water which was more pronounced at higher temperatures (Figs. 2.3, 2.4 and 2.5) due to the higher ammonification rate in the sediment and the higher rate of diffusion of ammonium from the sediment into the overlying water at higher temperatures. Without N-Serve, ammonium was converted to nitrate, except at 4°C where no significant nitrification occurred (Figs. 2.3, 2.4 and 2.5). It is likely that nitrification occurred mainly in the interface of the water-sediment system. The ultimate nitrification rate at 15 and 25°C was higher than the accumulation rate of ammonium in the overlying water (Fig. 2.4), which fits in with the normally low ammonium concentrations in natural waters. The lack of inhibition of heterotrophic nitrification by N-Serve and the absence of nitrate formation in the system treated with N-Serve indicate that this type of nitrification is of no importance in the conversion of ammonium to nitrate in water-sediment systems (Fig. 2.3).

Nitrate as well as nitrite supplied to the water-sediment system were removed from the overlying water at the three incubation temperatures (Figs. 2.4 and 2.5), but at 4°C a much longer lag period was observed than at 15 and 25°C. The denitrification rate of a water-sediment system was defined as the amount of nitrate or nitrite nitrogen (mg) consumed per square metre of sediment surface per day. The disappearance of nitrite was more rapid than that of nitrate at the three incubation temperatures tested (Fig. 2.6). Under similar circumstances, the denitrification rate occurring in sediment A was always higher than that in sediment B, because of differences in content of organic matter in the two sediments and availability of the organic matter as carbon source and hydrogen donor for denitrifying bacteria (Tables 2.1, 2.2 and 2.4 compared with Figs. 2.7, 2.8 and 2.9, respectively). The denitrification rate in sediment was adversely affected by the presence of oxygen in the overlying water. Above an oxygen concentration of 1.5–2 mg l^{-1}, no further decrease in denitrification rate occurred. (Fig. 2.7).

The denitrification rates in both types of sediment were dependent on nitrate concentrations in the overlying water, approaching first-order kinetics at lower concentrations, gradually becoming independent of nitrate concentration, as nitrate increased (Fig. 2.8). Empirically, the denitrification rates in both types of sediment approached Michaelis-Menten kinetics for nitrate, although the experimental conditions in the water-sediment system hardly complied with the terms of that theory. Maximum rates of denitrification were at a nitrate nitrogen concentration of 300 mg l^{-1} with sediment B and above 500 mg l^{-1} with sediment A. When starting with a nitrate nitrogen concentration of 25.2 mg l^{-1}, a sediment layer 7 mm thick with sediment A and 14 mm with B was roughly found to be involved in denitrification (Fig. 2.9). Exchange of nitrate between water and sediment was crucial for denitrification in the sediment. Denitrification rates in laboratory experiments would be considerably lower than those in natural environments according to a survey and discussion of the transport mechanisms (Chapter 2).

Denitrifying bacteria assimilate nitrogen for the synthesis of cell material (immobilization of nitrogen). The immobilized nitrogen may originate from nitrate, ammonium or organic nitrogen. During denitrification and during immobilization of nitrate, this compound disappears from natural waters. In immobilization, this is only temporary and inorganic nitrogen may re-appear in the natural waters after mineralization of cell material. Therefore, for the removal of nitrate from natural waters, denitrification is preferable to immobilization of nitrate. Chapter 3 contains the results of experiments with water-sediment systems in which it was investigated which part of the nitrogen, immobilized by denitrifying bacteria under denitrifying conditions, originated from nitrate and which part from other sources. The experiments were with labeled nitrogen compounds (^{15}N). Most of the added nitrate was lost as gases by denitrification: 97.2% with sediment A and 94.5% with sediment B (Table 3.3). The remaining part, 2.8 and 5.5%, respectively, was converted into organic nitrogen compounds and ammonia. In addition to nitrate nitrogen, ammonium nitrogen, present in the water-sediment, was immobilized by the denitrifying bacteria: 7.3 and 4.1% with sediments A and B, respectively, as percentage of the added nitrate nitrogen (Table 3.4). The proportion of inorganic nitrogen immobilized after addition of nitrate was equal to about 10% of the nitrate nitrogen added for both types of sediments.

The sequence of nitrogenous compounds, detected during denitrification in sediments,

was as follows: $NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$ (Figs. 4.3 and 4.5). The reduction of NO_3^- to NO_2^- was faster than the reduction of NO_2^- to N_2O, while the latter reduction step was faster than that of N_2O to N_2. In the presence of nitrate, the upper layer of sediment A turned grey-brown, while sediment B turned entirely from black to yellow-brown. This fading of both types of sediment presumably resulted from oxidation of sulphides and ferrous ions by denitrifying bacteria. The amount of faded sediment, and the course of the nitrate curves in the overlying water (Figs. 4.4 and 4.6) suggested that under the experimental conditions exhaustion of nitrate and depletion of available organic matter became the limiting factor for denitrification in sediments A and B, respectively. In the latter sediment, methane formation was inhibited by the presence of nitrate. In the absence of nitrate, 0.98 and 0.04 μmoles methane were formed per g dry sediment per day in sediments A and B, respectively.

In the natural environment, oxygen and nitrate diffused from the overlying water into the sediment. Oxygen respiration occurred mainly in the upper layer of sediment, while denitrification proceeded particularly in deeper layers where nitrate but no oxygen was present. When the available electron donors in the upper layer of sediment became exhausted, oxygen diffused into a deeper layer where electron donors were still available. This resulted in the shift of the zone of denitrification to a deeper layer. A water-sediment system consisting of a 3-cm thick layer of sediment A and overlying water of which the concentration of nitrate nitrogen was maintained at 25 mg l^{-1} and dissolved oxygen was kept above 6.1 mg l^{-1}, was used to follow the shift of the zone of denitrification as a function of time by measuring continuously the redox potential (Eh) in the sediment at three different depths (Fig. 5.4). The nitrate front in the sediment was plotted from the times when Eh showed a pronounced rise through penetration of NO_3^- to each depth. The oxygen front was plotted from the times at which Eh rose above +100 mV. From the results, the shift of the denitrification zone during incubation was deduced (Fig. 5.5). The speed of the denitrification zone in the sediment was 0.13 mm day^{-1} under the experimental conditions.

Population densities of different types of bacteria were highest in the upper 5 cm of the sediment (Fig. 6.1). The highest counts of heterotrophic denitrifying bacteria were at a depth of 2 cm below the water-sediment interface. At that depth, the number of heterotrophic denitrifiers as percentage of the total aerobic heterotrophic bacteria was also maximum (Fig. 6.2). The heterotrophic denitrifying population in sediment A under field conditions consisted of bacteria belonging to 8 genera, of which *Pseudomonas, Alcaligenes, Bacillus* and *Flavobacterium* were the most important (Table 6.4). During anaerobic incubation of the sediment in the presence of nitrate, an active denitrifying population developed (Tables 6.3 and 6.6) which included heterotrophic bacteria of only 4 genera, *Pseudomonas* and *Alcaligenes* being by far the most numerous (Table 6.5). Denitrifying *Bacillus* species, found in large numbers in the sediment under field conditions, did not play an important role in denitrification upon anaerobic incubation of the sediments in the presence of nitrate. Growth trials showed that the heterotrophic denitrifying population of the sediment could utilize many carbohydrates except cellulose (Table 6.7). Efforts to isolate *Thiobacillus denitrificans* from the sediment under field conditions and after anaerobic incubation in the presence of nitrate were unsuccessful. Only a few nitrate-reducing *Thiobacillus* strains were isolated.

Chapter 7 reports on the availability of inorganic and organic compounds in the

sediment as hydrogen donors for denitrifying bacteria. The contents of free amino acids in three sediments were very low compared to those of combined amino acids (Table 7.1). The amino acid patterns of the fraction of combined amino acids indicated that part of the amino acids in the sediments tested was perhaps present in some association with humic substances (Table 7.2). The 4.2 mg of carbohydrates found in 1 g of a batch of dry sediment A contained 43% hexoses, 27% pentoses and 29% uronic acids. The 2.6 mg of carbohydrates in 1 g of a dry sediment B batch contained 36% hexoses, 40% pentoses and 24% uronic acids (Table 7.4). Only a small part of the carbohydrates was present as water-soluble sugars. Roughly 55% of the carbohydrates in both sediments presumably consisted of hemicelluloses and 15% of cellulosic compounds (Table 7.4). The contents of organic matter of the two sediments (6% in A and 2% in B) and the amounts of free and combined amino acids, and carbohydrates suggested that the organic matter of both sediments consisted mainly of other compounds like lignic and humic substances (Tables 7.1, 7.3 and 7.4).

During incubation, carbohydrates in the anaerobic sediment were fermented (Table 7.7) mainly to acetic acid. In anaerobic water-sediment systems without nitrate, acetic acid accumulated in the anaerobic overlying water (Fig. 7.2) and was subsequently converted to methane by obligately anaerobic bacteria (Fig. 7.5). With nitrate present, acetic acid did not accumulate in the overlying water, although its formation was assumed. It was mainly consumed by denitrifying bacteria in the upper layer of sediment.

The observed accumulation of sulphate in the overlying water of an anaerobic nitrate-containing water-sediment system (Fig. 7.4) indicates that the fading of the black sediment to greyish brown was due to oxidation of black FeS by denitrifying bacteria.

It is concluded that carbohydrates, acetic acid and FeS are important hydrogen donors for oxygen-respiring and denitrifying bacteria in sediments. In a natural water-sediment system with oxygen and nitrate in the overlying water, denitrifying bacteria would be in a more privileged position than oxygen-respiring bacteria for supply with hydrogen donors from deeper layers of the sediment (Fig. 7.6).

The removal of nitrate from overlying water by denitrification in the underlying sediment was clearly shown in the laboratory experiments described in Chapters 2, 3, 4 and 5. The ultimate effect of this process in removing nitrate from natural waters under practical conditions depends on the rate of denitrification and on the ratio of water volume and area of sediment surface. The results of a field trial are given in Chapter 8, in which was studied the removal of nitrate from effluent of a waste water purification plant following discharge into a canal (Fig. 8.1). The loss of nitrate over a 800-m long reach of the canal was estimated by analysing daily water samples from four sample stations for 20 days (Fig. 8.4). Of the nitrate present in the canal water 56% disappeared during flow through the 800-m long reach. The average retention time of the canal water in that reach was 1.7 days. The average rate of nitrate nitrogen disappearance during 20 days was 537 mg m^{-2} day^{-1}. Laboratory experiments with undisturbed water-sediment profiles from the canal showed that the disappearance of nitrate was mainly caused by denitrification in the sediment (Fig. 8.6).

Chapter 9A describes a simple respirometer to measure simultaneously dissolved oxygen and nitrate in bacterial suspensions (Figs. 9A.1 and 9A.2). It proved to be an easy tool in denitrification studies (Figs. 9A.3 and 9A.4). The uptake of oxygen and nitrate by undisturbed cores was measured in a similar way (Fig. 9B.1). The results showed that

dissolved oxygen in the overlying water reduced the rate of denitrification in the sediment (Fig. 9B.2), an observation also made in Chapter 2 (Fig. 2.7). Measurements of the uptake of oxygen and that of nitrate by completely mixed suspensions of sediment showed that oxygen was taken up by sediment by chemical and biological processes, whereas nitrate was utilized only biologically.

By denitrification in aquatic sediments, the nitrogen content of shallow natural waters is decreased permanently and considerably. The information obtained in this study can do much to reduce concern for the consequences in eutrophication from discharge of nitrogen-containing waters into natural waters.

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By denitrification in aquatic sediments, the nitrogen content of shallow natural waters is decreased permanently and considerably. The information obtained in this study can do much to reduce concern for the consequences in eutrophication from discharge of nitrogen-containing waters into natural waters.

Samenvatting

Hoewel de moderne samenleving in Nederland een steeds grotere behoefte heeft aan schoon oppervlaktewater, vormen de dichte bevolking en de industrie een steeds grotere bedreiging voor het handhaven van een goede kwaliteit van dit oppervlaktewater. De behoefte om vervuiling van het oppervlaktewater te voorkomen wordt daarom steeds groter. Afvalwaterzuivering vormt slechts ten dele een oplossing omdat de anorganische voedingsstoffen, waaronder stikstof en fosfaat, meestal in grote hoeveelheden in het gezuiverde afvalwater achterblijven. Dit heeft tot gevolg dat een door deze anorganische verbindingen sterk bevorderde groei van algen (algenbloei) mogelijk is, hetgeen nare gevolgen heeft voor de kwaliteit van het ontvangende oppervlaktewater. Drainage-water van akkerbouwgebieden bevat soms vrij veel door een regenoverschot uitgespoeld nitraat, waardoor ook het lozen van drainage-water wordt beschouwd als een bijdrage tot de eutrofiëring van oppervlaktewater.

Het hier te bespreken onderzoek had tot doel de microbiologische omzettingen van stikstofverbindingen in oppervlaktewater te bestuderen, in het bijzonder voor zover ze verband houden met de verlaging van het stikstofgehalte van dit milieu. Van de in aanmerking komende processen werd vooral aan denitrificatie gedacht, omdat hierdoor nitraat (eindprodukt van de mineralisatie van organische N-verbindingen) in gasvormige N-produkten wordt omgezet die in de atmosfeer verdwijnen. Aangezien denitrificatie een anaëroob proces is en oppervlaktewater meestal aëroob is, werd gedacht aan de mogelijkheid van denitrificatie in het anaërobe sediment dat zich als regel op de bodem van oppervlaktewater bevindt.

Voor het bestuderen van de denitrificatie werd natuurlijk sediment met een bovenstaande laag water als model-systeem gebruikt in laboratoriumexperimenten. Hiervoor werden twee totaal verschillende typen sediment gebruikt. Het ene (A) was afkomstig uit een sloot waarop gezuiverd afvalwater van een kalvermesterij wordt geloosd, het andere (B) uit een sloot die drainage-water afvoert uit een akkerbouwgebied.

In hoofdstuk 2 worden de resultaten vermeld van proeven over ammonificatie, nitrificatie en denitrificatie in een water-sediment-systeem (met sediment A) bij 3 incubatietemperaturen (4, 15 en 25°C). Remming van de nitrificatie met N-Serve veroorzaakte ophoping van ammoniumstikstof in het bovenstaande water. Bij een verhoging van de incubatietemperatuur werd een toenemende ophoping van NH_4^+ waargenomen (Figuren 2.3, 2.4 en 2.5). Dit was een gevolg van de grotere ammonificatiesnelheid in het sediment en van de hogere snelheid van diffusie van het gevormde NH_4^+ vanuit het sediment naar het bovenstaande water. Zonder remstof vond nitrificatie plaats, behalve bij 4°C waarbij geen nitrificatie van enige betekenis was waar te nemen (Figuren 2.3, 2.4 en 2.5). Vermoedelijk kwam nitrificatie voornamelijk voor in het grensvlak van het water-sediment-systeem. Bij 15 en 25°C was de uiteindelijke nitrificatiesnelheid groter dan de accumulatiesnelheid van NH_4^+ in het bovenstaande water (Fig. 2.4), hetgeen in overeen-

stemming is met de lage gehaltes aan ammoniumstikstof die gewoonlijk in oppervlaktewateren worden gevonden. Uit het volledig afwezig zijn van nitraatproduktie bij aanwezigheid in het water-sediment-systeem van N-Serve (dat alleen de autotrofe nitrificatie onderdrukt), werd geconcludeerd dat in het genoemde systeem geen heterotrofe nitrificatie van enige betekenis voorkomt (Fig. 2.3).

Hoewel bij 4°C een veel langere 'lag-phase' bij de verdwijning van nitraat werd waargenomen dan bij 15 en 25°C, verdwenen bij alle drie de incubatietemperaturen de aan het water-sediment-systeem toegevoegde hoeveelheden nitraat en nitriet volledig uit het bovenstaande water (Figuren 2.4 en 2.5). De denitrificatiesnelheid werd gedefiniëerd als de hoeveelheid nitraat- of nitrietstikstof (mg) die wordt verbruikt per m^2 sedimentoppervlak per dag. Nitrietstikstof verdween steeds met een grotere snelheid uit het bovenstaande water van een water-sediment-systeem dan nitraatstikstof (Fig. 2.6). Onder vergelijkbare proefomstandigheden was de denitrificatiesnelheid in sediment A steeds hoger dan in B (Figuren 2.7, 2.8 en 2.9). Dit was het gevolg van verschillen in gehalte aan organische stof van de beide sedimenttypen (Tabellen 2.1, 2.2 en 2.4) en van verschillen in beschikbaarheid van de in het sediment aanwezige organische stof als koolstofbron en waterstofdonor voor denitrificerende bacteriën. De denitrificatiesnelheid in sediment werd ongunstig beïnvloed door de aanwezigheid van zuurstof in het bovenstaande water. Bij een zuurstofconcentratie hoger dan 1,5–2 mg l^{-1} nam de denitrificatiesnelheid niet verder af (Fig. 2.7).

In beide typen sediment was de denitrificatiesnelheid afhankelijk van de nitraatconcentratie in het bovenstaande water (Fig. 2.8). Empirisch benaderde de denitrificatiesnelheid in beide sedimenten de Michaelis-Menten-kinetiek voor nitraat als limiterend substraat, hoewel de proefomstandigheden nauwelijks voldeden aan de voorwaarden van deze theorie. De maximale denitrificatiesnelheid in sediment A lag bij een nitraatstikstofgehalte hoger dan 500 mg l^{-1} en in sediment B bij 300 mg l^{-1}. Bij een aanvangsconcentratie van 25,2 mg nitraatstikstof per liter in het bovenstaande water vond denitrificatie in sedimenten A en B alleen plaats in een bovenste laag sediment van respectievelijk 7 en 14 mm dikte (Fig. 2.9). Het transport van nitraat in het water-sediment-systeem speelt in dit onderzoek een grote rol. Aan het einde van hoofdstuk 2 zijn daarom de verschillende transportmechanismen voor nitraat opgesomd en besproken. Hieruit kan worden afgeleid dat de denitrificatiesnelheden zoals die worden gevonden in laboratoriumexperimenten aanmerkelijk lager zijn dan die in de natuur.

Denitrificerende bacteriën assimileren stikstof voor de synthese van celmateriaal (immobilisatie van stikstof). De geïmmobiliseerde stikstof kan afkomstig zijn van nitraat, ammonium of organische stikstofverbindingen. Gedurende denitrificatie en immobilisatie van nitraat verdwijnt dit uit het oppervlaktewater, maar in het geval van immobilisatie is dit slechts tijdelijk en na mineralisatie van het celmateriaal kan de stikstof weer in het oppervlaktewater terecht komen. Daarom is denitrificatie een gunstiger proces voor de verwijdering van nitraat uit oppervlaktewater dan immobilisatie van nitraat. Hoofdstuk 3 bevat de resultaten van proeven met water-sediment-systemen waarin werd onderzocht welk gedeelte van het aanwezige nitraat onder denitrificerende omstandigheden werd gebruikt voor de celsynthese van denitrificerende bacteriën. In beide sedimenten A en B, vervluchtigden respectievelijk 97,2 en 94,5% van het toegevoegde nitraat door denitrificatie en werden slechts respectievelijk 2,8 en 5,5% omgezet in organische stikstofverbindingen en ammonia (Tabel 3.3). Naast nitraatstikstof immobiliseerden de denitrificerende

bacteriën ook nog ammoniumstikstof, namelijk 7,3 en 4,1% (uitgedrukt als percentage van de toegevoegde hoeveelheid nitraatstikstof) in respectievelijk sediment A en B (Tabel 3. 4). De totale hoeveelheid anorganische stikstof die in beide typen sediment werd geïmmobiliseerd als gevolg van de toevoeging van nitraat kwam hierdoor op ongeveer 10% van de toegevoegde hoeveelheid nitraatstikstof.

De opeenvolging van de verschillende stikstofverbindingen die tijdens denitrificatie in sedimenten werden aangetoond was als volgt: $NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$ (Figuren 4.3 en 4.5). De reductie van NO_3^- naar NO_2^- verliep sneller dan de reductie van NO_2^- naar N_2O, terwijl deze laatste reductiestap weer sneller was dan die van N_2O naar N_2. In aanwezigheid van nitraat veranderde de zwarte kleur van sediment A in grijsbruin, terwijl die van sediment B veranderde in geelbruin. Deze verkleuring werd blijkbaar veroorzaakt door de oxydatie van S^{2-} en Fe^{2+} door denitrificerende bacteriën. Uit de mate van verkleuring van de sedimenten en uit het verloop van het nitraatverbruik (Figuren 4.4 en 4.6) werd geconcludeerd dat onder de proefomstandigheden het nitraat de beperkende factor was voor de denitrificatie in sediment A, terwijl in sediment B dit de beschikbare organische stof was. In sediment B werd de vorming van methaan door de aanwezigheid van nitraat geremd. Bij afwezigheid van nitraat waren de snelheden waarmee methaan werd gevormd in sediment A en B respectievelijk 0,98 en 0,04 μmoles per g sediment droge stof per dag.

Onder natuurlijke omstandigheden diffundeerde behalve nitraat ook zuurstof vanuit het bovenstaande water in het sediment. Verbruik van zuurstof vond voornamelijk plaats in de bovenste laag van het sediment, terwijl denitrificatie plaats vond in de daaronder gelegen laag waar geen zuurstof maar wel nitraat aanwezig was. Als de beschikbare elektronendonors in de bovenste laag van het sediment uitgeput raakten, drong zuurstof dieper in het sediment door naar plaatsen waar deze donors nog wel beschikbaar waren. Dit had verschuiving van de denitrificatiezone naar een dieper in het sediment gelegen plaats tot gevolg. De snelheid van verplaatsing van de denitrificatiezone werd indirect gemeten in een kolom bestaande uit een 3-cm dikke laag sediment A waarboven zich water bevond. Het nitraatstikstofgehalte werd gedurende de proef op 25 mg l^{-1} en het zuurstofgehalte boven de 6,1 mg l^{-1} gehouden. De verschuiving van de denitrificatiezone met de tijd werd indirect vervolgd door het meten van de redoxpotentiaal (Eh) in het sediment op drie verschillende diepten (Fig. 5.4). Het tijdstip waarop de Eh op een bepaald meetpunt begon te stijgen gaf aan dat het nitraatfront deze diepte had bereikt. Als maat voor het bereiken van een bepaalde diepte door zuurstof werd het tijdstip gekozen waarop de Eh boven +100 mV was gestegen. Doordat de Eh op 3 verschillende diepten in het sediment werd gemeten, kon de penetratie van het nitraat- en zuurstoffront in het sediment als functie van de tijd worden afgeleid (Fig. 5.5). Uit deze resultaten werd de plaats van de denitrificatiezone gedurende de gehele incubatieperiode afgeleid (Fig. 5.5). De denitrificatiezone bleek onder deze omstandigheden het sediment binnen te dringen met een snelheid van 0,13 mm/dag.

De in hoofdstuk 6 vermelde uitkomsten betreffende de in sediment voorkomende bacteriën laten zien dat de grootste aantallen werden gevonden in de bovenste 5 cm van het sediment (Fig. 6.1). Het grootste aantal heterotrofe denitrificerende bacteriën, zowel absoluut als in % van het totale aantal heterotrofe aërobe bacteriën, kwam voor op een diepte van 2 cm (Figuren 6.1 en 6.2). Onder natuurlijke omstandigheden bestond de populatie van heterotrofe denitrificerende bacteriën in sediment A uit vertegenwoordigers van 8 verschillende geslachten, waarvan *Pseudomonas, Alcaligenes, Bacillus* en *Flavobac-*

terium het sterkst vertegenwoordigd waren (Tabel 6.4). Tijdens anaërobe incubatie van het sediment in aanwezigheid van nitraat kwam een actief denitrificerende bacteriepopulatie tot ontwikkeling (Tabellen 6.3 en 6.6), waarvan de vertegenwoordigers slechts tot 4 geslachten behoorden, waarvan *Pseudomonas* en *Alcaligenes* verreweg het belangrijkst waren (Tabel 6.5). Uit dit onderzoek bleek dat de oorspronkelijk gevonden denitrificerende *Bacillus*-soorten een niet zo belangrijke rol bij de denitrificatie in water-sediment-systemen speelden als hun aantal in het sediment deed voorkomen. In groeiproeven werd aangetoond dat de populatie van heterotrofe denitrificerende bacteriën uit het sediment vele koolhydraten kon benutten (Tabel 6.7), behalve cellulose. Uit natuurlijk sediment en uit sediment na anaërobe incubatie in aanwezigheid van nitraat kon geen *Thiobacillus denitrificans* geïsoleerd worden. Er werden wel enkele *Thiobacillus*-stammen verkregen die nitraat alleen tot nitriet konden reduceren.

In hoofdstuk 7 worden gegevens vermeld over de in sediment voorkomende anorganische en organische verbindingen die als waterstofdonor kunnen dienen voor denitrificerende bacteriën. De gehaltes aan vrije aminozuren in 3 monsters sediment waren erg laag vergeleken met die aan gebonden aminozuren (Tabel 7.1). Het aminozuurpatroon van dē fractie gebonden aminozuren wees erop dat een deel van de aminozuren in de geteste sedimenten mogelijk gebonden was aan humusverbindingen (Tabel 7.2). De in 1 g van een bepaald monster droog sediment A gevonden 4,2 mg koolhydraten bestond voor 43% uit hexosen, 27% uit pentosen en 29% uit uronzuren. Een zelfde hoeveelheid sediment B bevatte 2,6 mg koolhydraten voor 36% bestaande uit hexosen, 40% uit pentosen en 24% uit uronzuren (Tabel 7.4). Slechts een klein gedeelte van deze koolhydraten in beide sedimenten was in water oplosbaar. Ongeveer 55% van de koolhydraten in beide sedimenten was vermoedelijk aanwezig als hemicellulosen en 15% als celluloseachtige verbindingen (Tabel 7.4). Uit de in beide sedimenten gevonden hoeveelheden organische stof, aminozuren en koolhydraten werd geconcludeerd dat de organische fractie van sediment hoofdzakelijk bestond uit verbindingen zoals lignine en humuszuren (Tabellen 7.1, 7.3 en 7.4).

Bij incubatie van anaëroob sediment werden de assimileerbare koolhydraten vergist (Tabel 7.7) waarbij azijnzuur als belangrijkste eindprodukt ontstond. Bij afwezigheid van zuurstof en nitraat accumuleerde azijnzuur in het anaërobe bovenstaande water (Fig. 7.2). Dit zuur werd daarna door obligaat anaërobe bacteriën omgezet in methaan (Fig. 7.5). In aanwezigheid van nitraat vond geen accumulatie van azijnzuur in het bovenstaande water plaats, hoewel vast stond dat het gevormd werd (Fig. 7.2). In dit geval werd het azijnzuur hoofdzakelijk geconsumeerd door denitrificerende bacteriën in de bovenlaag van het sediment.

De ophoping van sulfaat in het bovenstaande water van een anaëroob water-sediment-systeem in aanwezigheid van nitraat (Fig. 7.4) wijst erop dat de verkleuring van het sediment van zwart naar bruinachtig berustte op de oxydatie van FeS door denitrificerende bacteriën.

Uit de in hoofdstuk 7 beschreven resultaten wordt geconcludeerd dat koolhydraten, azijnzuur en FeS kunnen worden beschouwd als belangrijke waterstofdonors voor ademhalende en denitrificerende bacteriën in sedimenten. In natuurlijke water-sediment-systemen met zuurstof en nitraat aanwezig in het bovenstaande water zijn denitrificerende bacteriën zeer waarschijnlijk in het voordeel boven zuurstofgebruikende bacteriën bij het verkrijgen van de genoemde waterstofdonors afkomstig uit diep gelegen lagen van

het sediment (Fig. 7.6).

De verdwijning van nitraat uit het bovenstaande water van water-sediment-systemen als gevolg van denitrificatie in het sediment is in laboratoriumproeven duidelijk aangetoond (Hoofdstukken 2, 3, 4 en 5). Het uiteindelijke effect van dit proces op het stikstofgehalte van oppervlaktewater hangt af van de denitrificatiesnelheid en de verhouding tussen het watervolume en het sedimentoppervlak. In een veldexperiment is geprobeerd dit effect aan te tonen (Hoofdstuk 8). Hierbij werd de verdwijning van nitraat uit effluent van een zuiveringsinstallatie bestudeerd na lozing van het effluent op een kanaal (Fig. 8.1). Gedurende 20 dagen werd in een 800 m lang traject van het kanaal het nitraatverlies bepaald door het analyseren van dagelijks genomen monsters afkomstig van 4 bemonsteringsplaatsen in dit traject (Fig. 8.4). Van het nitraat dat bij het begin van het 800 m lange traject in het kanaalwater aanwezig was, verdween 56% gedurende het doorstromen van het traject (Tabel 8.2). De gemiddelde verblijftijd van het water in het traject was 1,7 dagen. De gemiddelde snelheid waarmee het nitraat verdween, uitgerekend over de periode van 20 dagen, bedroeg 537 mg NO_3^--N m^{-2} day^{-1}. Laboratoriumproeven met ongestoorde water-sedimentprofielen uit het kanaal toonden aan, dat de bovengenoemde verdwijning van nitraat werd veroorzaakt door denitrificatie (Fig. 8.6).

In hoofdstuk 9A is een eenvoudige respirometer voor het gelijktijdig meten van opgeloste zuurstof en nitraat beschreven (Figuren 9A.1 en 9A.2). Dit apparaat biedt mogelijkheden om op snelle wijze informatie te verkrijgen over zuurstofademhalings- en denitrificatiesnelheden in volledig gemengde systemen (Figuren 9A.3 en 9A.4). De opname van zuurstof en nitraat door ongestoorde sedimentprofielen werd op een soortgelijke manier gemeten (Fig. 9B.1). De resultaten toonden aan dat opgeloste zuurstof in het bovenstaande water de denitrificatiesnelheid in het sediment verminderde (Fig. 9B.2), hetgeen ook in hoofdstuk 2 was waargenomen (Fig. 2.7). Uit metingen van de zuurstof- en nitraatopname in volledig gemengde sedimentsuspensies werd geconcludeerd dat in sediment zuurstof zowel in chemische als in biologische processen werd verbruikt, terwijl de opname van nitraat door het sediment alleen plaats vond onder invloed van biologische processen.

Dit onderzoek heeft aangetoond, dat het stikstofgehalte van ondiepe oppervlaktewateren voortdurend en in belangrijke mate wordt verlaagd door het optreden van denitrificatie in sedimenten. Een juiste informatie over dit verschijnsel kan veel bezorgdheid over eutrofiëringsproblemen als gevolg van de lozing van stikstofhoudende verbindingen doen wegnemen.

Verantwoording

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Curriculum vitae

In 1966 behaalde de op 17 januari 1948 te Dodewaard geboren auteur het HBS-B diploma aan het Heldring College te Zetten. Na zijn studie in de waterzuivering aan de Landbouwhogeschool te Wageningen gedurende september 1966 – juni 1972, is hij in september 1972 begonnen aan een promotie-onderzoek in het Laboratorium voor Microbiologie van de Landbouwhogeschool.