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

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Abstract

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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.

This thesis will be published as Agricultural Research Reports 858.

Stellingen

1
Het stikstofgehalte van ondiepe oppervlaktewateren wordt voortdurend en in belangrijke mate verlaagd door in het sediment voorkomende denitrificatie. Informatie over dit verschijnsel kan veel bezorgdheid over eutrofiëringsproblemen als gevolg van stikstofhoudende verbindingen wegnemen.

Dit proefschrift

2
De door Davies verkregen resultaten betreffende de verwijdering van nitraat uit effluent van zuiveringsinstallaties met behulp van denitrificerende bacteriën die methaan als waterstofdonor zouden gebruiken, zijn twijfelachtig.

T. R. Davies, 1973. Water Research 7: 575–579.

3
Het is te verwachten dat in de toekomst de simultane fosfaatverwijdering bij de afvalwaterzuivering zal worden vervangen door een naprecipitatie.

J. Leentvaar, 1974, H_2O 7: 370–372.

4
Bij het ontwerpen van rioolstelsels zouden in verband met het al of niet toelaten van overstortingen, gefundeerde kwaliteitseisen voor het oppervlaktewater in het te rioleren gebied uitgangspunt moeten zijn.

5
Het huidige veilingsysteem is een te passief instrument voor een goede afzet van hard fruit; de vorming van een centrale afzetcoöperatie met verstrekkende inspraakmogelijkheden in de gehele fruitteelt zal een betere garantie bieden voor deze afzet.

A. D. Little, 1971. Action program for apples and pears. Ministerie van Landbouw en Visserij.

6
Voor een milieubewuste gewasbescherming in de fruitteelt is een verdere ontwikkeling van 'geïntegreerde' bestrijdingsmethoden noodzakelijk. Gezien de toenemende afkeer van de consument van veelvuldig met chemische bestrijdingsmiddelen behandelde produkten enerzijds en de mogelijke kostenbesparingen voor de teler anderzijds, dient vooralsnog omgeschakeld te worden van 'schematische' naar 'geleide' bestrijdingsmethoden.

Tijdschrift van de Nationale Raad voor Landbouwkundig Onderzoek-TNO, 1974. Nr. 5: 14–18.

7

De jaarlijkse uitbarsting van botulisme onder watervogels buiten de stedelijke gebieden vindt zijn oorzaak niet in bepaalde klimatologische en hydrologische omstandigheden, maar in het onvoldoende aanwezig zijn van natuurlijke vijanden van watervogels en aaseters.

J. Haagsma, 1973. Proefschrift, Rijksuniversiteit Utrecht.

8

In het milieubeheer zijn recirculatiesystemen alleen dan zinvol wanneer zij gepaard gaan met een laag energieverbruik.

Proefschrift van J. F. van Kessel
Influence of denitrification in aquatic sediments on the nitrogen content of natural waters
Wageningen, 8 oktober 1976

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.

Introduction

The major transformations of nitrogen carried out by bacteria are nitrification, denitrification, N_2-fixation, ammonification and immobilization. All these processes may occur in a water-sediment system. A schematic diagram of the coherence between these processes in a water-sediment system in the natural environment is given in Fig. 2.1. The external nitrogen sources in this figure include sewage, effluent of purification plants, drainwater of agricultural areas and rain water. The extent of these external sources has been increased during the last decennia. The introduction of waste water purification has resulted in disposal of ammonium and nitrate as far as nitrogen is concerned.

Disappearance of nitrogen in natural waters can be due to bacterial reduction of nitrate. Many types of aerobic heterotrophic bacteria can use nitrate as the terminal electron acceptor under anaerobic conditions (Payne, 1973). Nitrate reduction during this process can proceed to different compounds, *viz.* nitrite, nitric oxide, nitrous oxide, molecular nitrogen or ammonium. The formation of gaseous products from nitrate or nitrite is called denitrification. Wuhrmann & Mechsner (1966) showed that denitrification was strongly impaired by oxygen at approximate neutrality. Natural waters generally contain dissolved oxygen in such a concentration, that denitrification in the overlying water of sediment is almost impossible. However, sediments are highly reduced and rich in organic matter, so that here the circumstances for denitrification are favourable. Denitrification in sediments has already been shown in Lake Mendota by Chen *et al.*

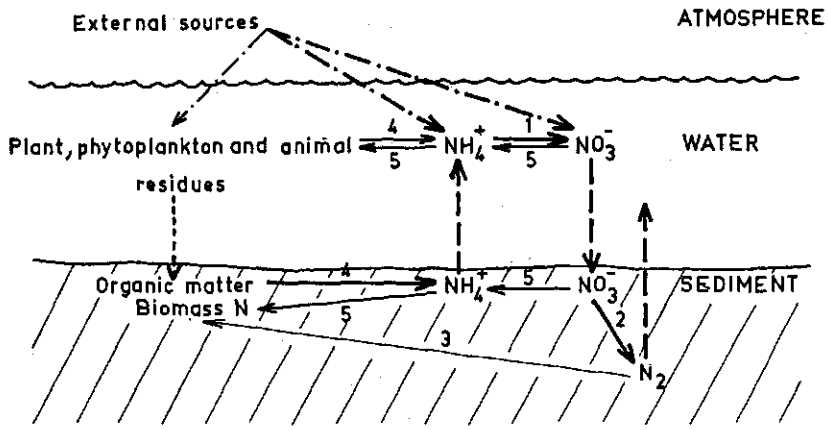

Fig. 2.1. The major transformations of nitrogen in a water-sediment system.
(1) Nitrification; (2) Denitrification; (3) N_2-fixation; (4) Ammonification; (5) Immobilization.
------ Sedimentation; – – – Diffusion; – . – Disposal.
The thickness of the lines gives an impression concerning the importance of the processes.

(1972a) and in the Pacific off Peru by Goering & Pamatmat (1971).

In the present study the factors affecting the rate of denitrification were tested with two types of sediments. The factors studied were temperature, dissolved oxygen and nitrate concentration in the water above the sediment, and thickness of the sediment layer.

Materials and methods

Sediments

Two types of sediments, A and B, were used. Type A originated from the 10-cm upper layer of a ditch into which effluent of a purification plant was discharged. This plant consisted of an activated sludge aeration tank and a final clarification tank. The plant was only fed with slurry from calfs destinated for meat production. Type B originated from the 10-cm upper layer of a ditch into which water from arable land was drained off.

The wet sediments were passed through a sieve with meshes of 3 mm diameter, to remove large stones, and subsequently they were well homogenized. Some chemical data concerning the sediments are presented separately for each factor studied in the section 'Results and Discussion' (Tables 2.1, 2.2 and 2.4), because in each case a new amount of sediment was taken from the ditches.

The experiments were carried out in the dark to eliminate the immobilization of inorganic nitrogenous compounds by phytoplankton.

Experiments concerning the influence of temperature on the denitrification rate

Glass tubes with an inner diameter of 3.6 cm were filled with about 60 g of wet sediment type A. A perforated plate having a diameter of 3.1 cm was laid on the surface

of the sediment enabling the addition of a liquid without disturbing the sediment. The tubes were divided into 6 series to which 100 ml neutral aqueous solution was added containing no solutes, 25 mg $NaNO_3$-N, 25 mg $NaNO_2$-N, 5 mg N-Serve, 25 mg $NaNO_3$-N plus 5 mg N-Serve and 25 mg $NaNO_2$-N plus 5 mg N-Serve per litre, respectively. After these additions the perforated plates were carefully removed without disturbing the surface of the sediment. The N-Serve (2-chloro-6-(trichloromethyl)-pyridine) was added to inhibit nitrification (Sommer, 1972; Bundy & Bremner, 1973). The oxidation of nitrite is not inhibited by this compound (Campbell & Aleem, 1965). The N-Serve did not appear to be toxic in soil for other microorganisms in concentrations of 1000–1200 ppm (Young, 1973). In preliminary experiments it appeared that for a complete inhibition of the ammonia-oxidizing bacteria re-addition of N-Serve was necessary every other week. Keeney (1973) mentioned that in a Lake Mendota sediment sample, nitrification stopped entirely for 7 days after addition of N-Serve, while Sommer (1972) even observed a complete inhibition for 56 days of incubation at 25°C after an addition of 5 ppm N-Serve to soil.

The tubes of each series were incubated at 4, 15 and 25°C, respectively, except those with the addition of 100 ml aqueous solution containing no solutes and 5 mg N-Serve per litre, which were not incubated at 15°C. The experiment was carried out in duplicate. The pH and the concentrations of nitrate, nitrite and ammonium nitrogen in the overlying water were determined several times during the incubation period. The results were corrected for the increase of concentration caused by evaporation of water during the experimental period. Therefore, the tubes were weighed at regular times during the incubation period.

Experiments concerning the influence of oxygen on the denitrification rate

Six glass tubes with an inner diameter of 4.8 cm were filled with about 200 g of wet sediment type A and 6 other tubes with the same amount of wet sediment type B. Above the sediment 200 ml of a neutral aqueous solution containing 25 mg $NaNO_3$-N and 5 mg N-Serve per litre were pipetted without disturbing the surface of the sediment (see the foregoing experiment). A gradient of dissolved oxygen concentrations in the overlying water of each series of 6 tubes was established by bubbling different gas mixtures through the overlying water of the tubes during the incubation period (Fig. 2.2). The gas mixtures were obtained from cylinders containing molecular nitrogen with 0, 2.5, 5, 10, 15 and 21% oxygen, respectively. The gas was first led through the overlying water in the tube with sediment type B and subsequently through the tube with sediment type A. After passing the tube with A, the gas stream was led through a waterseal to prevent a reverse stream of air. The flow rates of the various gas mixtures bubbling through the water were equal and they maintained oxygen concentrations of 0, 1.0, 2.1, 4.1, 6.2 and 8.7 mg l^{-1}, respectively, in the overlying water. At the flow rates used, the sediment was not disturbed. The sediments were incubated at 25°C during 11 days. This experiment was repeated three times. The amount of evaporated water during the experimental period was made up at the end of this period after which the contents of nitrate, nitrite and ammonium nitrogen in the overlying water were determined and compared with those at the beginning of the incubation.

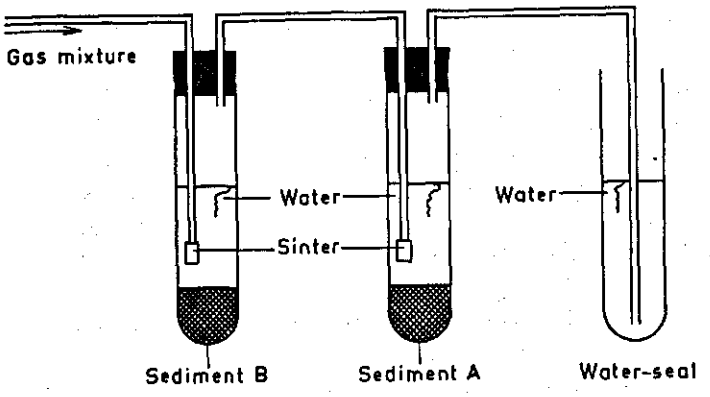

Fig. 2.2. Design to establish various dissolved oxygen concentration levels in the overlying water of the sediment during incubation.

Experiments concerning the influence of various nitrate levels on the denitrification rate

Six glass tubes with an inner diameter of 4.8 cm were filled with about 200 grams of wet sediment. Above the sediment 100 ml neutral aqueous solution was carefully pipetted without disturbing the surface of the sediment (as before). This solution contained besides 5 mg N-Serve also 0 (blank), 5.2, 25.6, 51, 103 and 500 mg sodium nitrate nitrogen per litre, respectively. After 3 days of incubation at 25°C the concentrations of nitrate, nitrite and ammonium nitrogen in the overlying water were determined. Subsequently, the solution of each tube was sucked off and replaced by 100 ml solution containing the same amounts of nitrate nitrogen and N-Serve as supplied at the beginning of the experiment. This procedure was repeated 5 times. The experiment was carried out in duplicate with both types of sediments.

Experiments concerning the influence of the sediment layer thickness on the denitrification rate

Polymethyl metacrylate tubes with a flat bottom and an inner diameter of 3.4 cm were filled with layers of sediment of different thicknesses. With sediment type A these layers were 2, 5, 7.5, 12, 18 and 30 mm thick and with sediment type B, 3, 7, 12, 17.5, 28 and 44 mm. Above the sediment 50 ml of a neutral aqueous solution containing 25.2 mg $NaNO_3$-N and 5 mg N-Serve per litre was carefully pipetted without disturbing the surface of the sediment. The tubes were incubated at 25°C. Nitrate, nitrite and ammonium nitrogen in the overlying water were determined daily. The experiment was repeated once for both types of sediments.

Chemical analysis

Nitrate nitrogen was determined by the salicylate method described by Müller & Widemann (1955). Nitrite nitrogen was determined by the method of Griess-Romijn van Eck (NEN 3235 6.3, 1972). The ammonium nitrogen present in the overlying water was

determined by the Nessler method (Deutsche Einheitsverfahren, 1960). The ammonium and organic nitrogen in the sediment were estimated according to Bremner (1965).

Results and discussion

The influence of temperature on nitrification and denitrification

Some chemical data concerning sediment A, used in this experiment, are presented in Table 2.1. During incubation the overlying water of all of the water-sediment systems contained permanently more than 3–5 mg l^{-1} dissolved oxygen and the pH of the overlying water varied between 7.0 and 7.8. Nitrate and nitrite did not accumulate in the water above the sediment during incubation at 4°C (Fig. 2.3). Since the ammonium curves do not differ significantly in the absence and presence of N-Serve (which inhibits nitrification), it can be concluded that significant nitrification did not occur at 4°C. This result is in agreement with the statement of Painter (1970) that at low temperatures no or little growth of nitrifying bacteria occurs.

At 25°C the ammonium-nitrogen content in the water of the tubes without N-Serve increased during the first 20 days of incubation while in the presence of N-Serve the ammonium-nitrogen content continued to increase after 20 days (Fig. 2.3). The higher values of ammonium nitrogen at 25°C than at 4°C were due to the higher ammonification rate and the higher diffusion rate of ammonium from the sediment into the overlying water at 25°C. In the tubes without N-Serve added, the ammonium content decreased after 20 days of incubation which corresponded with the formation of nitrite. The latter reached a maximum at the 33rd day of incubation after which the main part of the nitrite was converted to nitrate and the remainder was probably removed by denitrification. The maximum amount of nitrate nitrogen was found at the 42nd day. During the last 20 days of the incubation period nitrate disappeared while the ammonium-nitrogen content increased. This means that during this period the rate of ammonium accumulation in the water above the sediment was higher than the nitrification rate.

The results obtained with nitrate added to the system incubated at 4°C and 25°C were almost the same as those without added nitrate, except for the disappearance of the added nitrate (Fig. 2.4).

With added nitrite and incubated at 25°C, the ammonium, nitrite and nitrate peaks were reached after 15, 21 and 30 days, respectively, contrasting with the results obtained with the systems treated with nitrate or without added nitrogen (compare Fig. 2.5 with Figs. 2.3 and 2.4). In the nitrate and no nitrogen tubes these peaks were reached after 20, 33

Table 2.1. Some chemical data of sediment A used in the experiment on the influence of temperature (mg per g wet weight).

Dry weight	680
Organic matter determined as volatile solids	33.5
Ammonium nitrogen	0.78
Organic nitrogen	0.91
pH	7.65

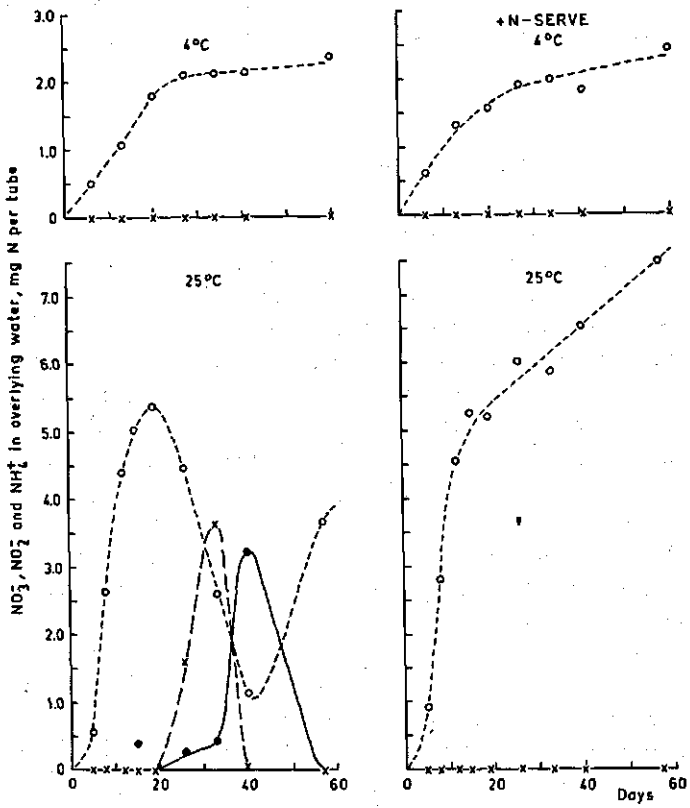

Fig. 2.3. Nitrate, nitrite and ammonium nitrogen in the overlying water of water-sediment systems incubated at 4 and 25°C in the absence and presence of N-Serve.
—●— NO_3^--N; – x – NO_2^--N; - -o- - NH_4^+-N.

and 42 days, respectively. No explanation of these differences can be given. The nitrate-nitrogen content of the nitrite-treated systems was rather high after 60 days of incubation at 25°C in comparison with the other treated systems.

Directly after the start of the incubation period, small amounts of nitrate were detected in the overlying water containing nitrite (Fig. 2.5). This apparent nitrate did not originate from the nitrification process but was the result of the well-known interference of nitrite with the determination of nitrate (Müller & Widemann, 1955).

At 15°C the increase of the ammonium-nitrogen content in the overlying water of the nitrate and nitrite-treated systems turned into a decrease after about 20 days of incubation (Figs. 2.4 and 2.5). In the nitrate-treated system a nitrite peak was found at the 40th day of incubation, whereas in the nitrite-treated system no nitrite was found after the consumption of the added nitrite. The nitrate-nitrogen content of the nitrate-treated systems increased after having fallen to nearly zero, from the 30rd day until the end of the experiment, whereas in the nitrite-treated systems only a small peak was found at the 45th day.

In the systems supplied with nitrate plus N-Serve, no nitrite was detectable during the

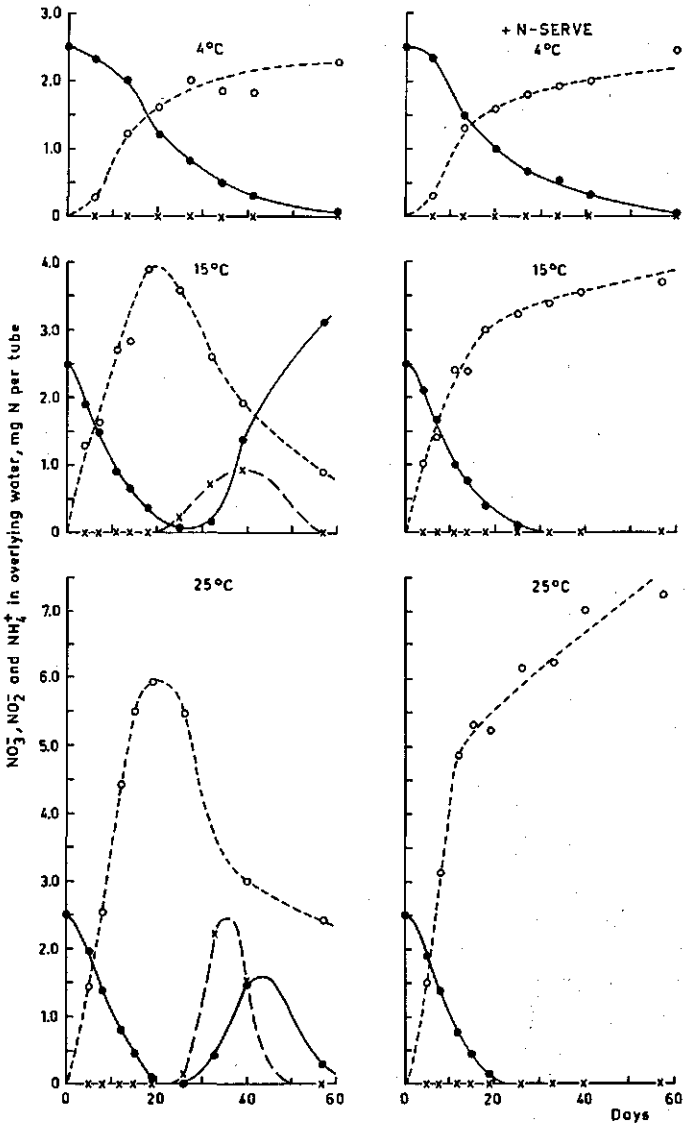

Fig. 2.4. The effect of temperature on the nitrate, nitrite and ammonium-nitrogen contents in the overlying water of water-sediment systems supplied with 2.5 mg NO_3^--N per tube in the absence and presence of N-Serve.
—•— NO_3^--N; – x – NO_2^--N; - -o- - NH_4^+-N.

incubation period. This means that in the systems supplied with nitrate only (no N-Serve), the accumulated nitrite originated from nitrification and not from denitrification (Fig. 2.4). The observed nitrite accumulation suggests that the oxidation of nitrite to nitrate by *Nitrobacter* was inhibited by the presence of ammonia in the system. Inhibition of nitrite oxidation in a pure culture of *Nitrobacter agilis* by ammonium salts

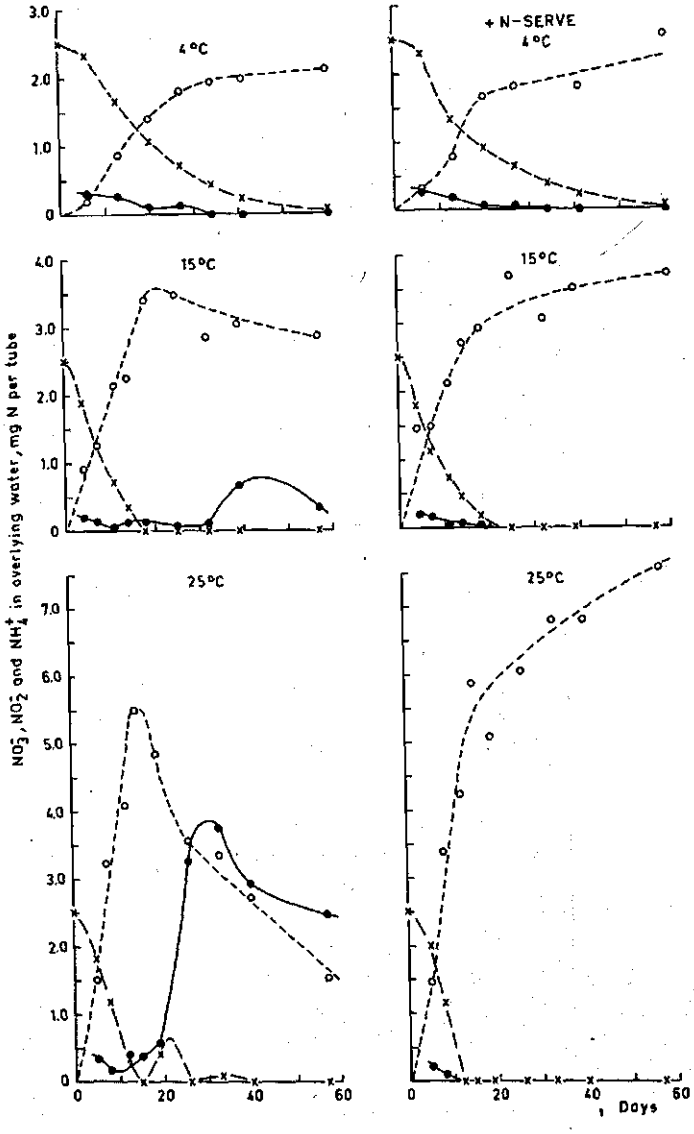

Fig. 2.5. The effect of temperature on the nitrate, nitrite and ammonium-nitrogen contents in the overlying water of water-sediment systems supplied with 2.5 mg NO_2^--N per tube in the absence and presence of N-Serve.
—•— NO_3^--N; – x – NO_2^--N; - -o- - NH_4^+-N.

in alkaline solution was observed by Aleem & Alexander (1960), while Nakos & Wolcott (1972) from their experiments with a mixed culture of *Nitrosomonas europaea* and *Nitrobacter agilis* concluded that the nitrite accumulation was due to a bacteriostatic effect of ammonium ions or ammonia on the growth of *Nitrobacter agilis*.

At 25°C nitrate and nitrite were not detectable in the overlying water of water-

sediment systems only supplied with N-Serve, whereas both compounds were present in the overlying water of the water-sediment systems without any addition after 20 days of incubation (Fig. 2.3). This observation and the statement of Shattuck & Alexander (1963) that N-Serve does not inhibit heterotrophic nitrification indicate that heterotrophic nitrification, if occurring at all, did not provide an important contribution to the nitrification process in sediment A. Chen *et al.* (1972b) came to the same conclusion from nitrification studies with sediments of Lake Mendota.

In an earlier study it was shown that the disappearance of added nitrate and nitrite from the overlying water was mainly caused by denitrification. A water-sediment system with the same type of sediment lost 97.2% of the added nitrate by volatilization due to denitrification (Chapter 3). In all experiments of the present study, the remaining part of the disappeared nitrate, although converted into organic and ammonium nitrogen, is for convenience considered as belonging to the denitrified nitrate. It is likely, that denitrification occurred only in the anaerobic sediment, as the overlying water contained dissolved oxygen. Any dentrification occurring in anaerobic microzones of the overlying water (Brezonik, 1975) was supposed to be unlikely, because the overlying water in all experiments of the present study did not contain particles to form such anaerobic microzones. In a preliminary experiment it was found that the nitrate content of the interstitial water of sediments was always nil, in spite of nitrate being present in the overlying water.

The supplied nitrate as well as nitrite were denitrified at the three incubation temperatures used (Figs. 2.4 and 2.5). The lag period of denitrification at 4°C was much longer than that at 15 and 25°C. In denitrification studies with a pure culture of *Pseudomonas denitrificans*, Dawson & Murphy (1972) found also a decreasing lag period with an increasing temperature *viz.*, from about 8 days at 5°C to about 8 h at 27°C. After the lag period an almost linear drop of the nitrate as well as the nitrite content in the overlying water was observed down to a concentration of about 10 mg l^{-1} nitrate or nitrite nitrogen (1.0 mg per tube) at the used incubation temperatures. The denitrification rate of the systems with nitrate plus N-Serve (mg NO_3^--N m^{-2} day^{-1}) was calculated at each incubation temperature from the time (days) needed for consuming 1.0 mg nitrate nitrogen, and from the area of the sediment surface (m^2). For this calculation the linear decline of the nitrate-nitrogen content from 2.0 to 1.0 mg per tube was used (Fig. 2.4). In the same way the denitrification rates with nitrite plus N-Serve added were computed from Fig. 2.5. For both electron acceptors these denitrification rates were plotted versus the corresponding incubation temperatures (Fig. 2.6). The denitrification rate with both electron acceptors increased directly proportionally to the temperature between 4 and 25°C (Fig. 2.6). At each temperature the rate of nitrite reduction was always higher than that of nitrate. A similar result was obtained in a water-saturated soil (Bailey & Beauchamp, 1973) and in concentrated waste water (Prakasam & Loehr, 1972). An obvious explanation might be that for the anaerobic respiration of a certain amount of substrate less nitrate than nitrite is needed, because nitrate accepts 5 and nitrite only 3 electrons per ion during reduction to molecular nitrogen. Another explanation might be that nitrite was reduced not only by nitrite-reducing bacteria, which were incapable of reducing nitrate dissimilatory, but also by nitrate-reducing bacteria. Organisms of the former type were isolated by Pichinoty & Chatelain (1973) and Vangnai & Klein (1974).

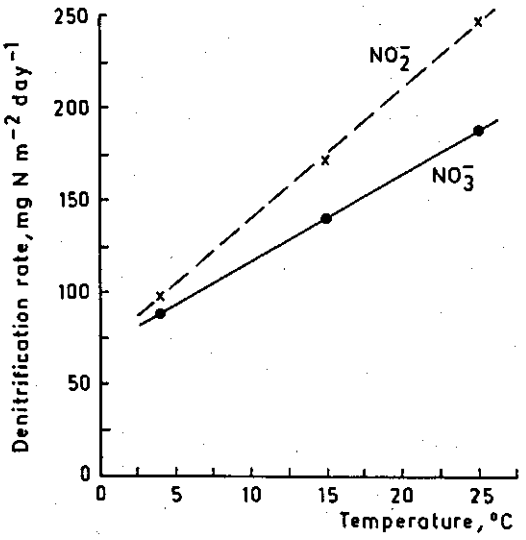

Fig. 2.6. The relation between incubation temperature and denitrification rate of a water-sediment system containing nitrate or nitrite.

The influence of the dissolved oxygen concentration on the denitrification rate

Some chemical data concerning sediments A and B, used for this experiment, are presented in Table 2.2. At the start of the incubation period 5 mg nitrate nitrogen was present in the overlying water of both types of sediment.

The amount of nitrate nitrogen present in the overlying water after 260 hours of incubation at 25°C was generally higher when the dissolved oxygen concentration increased (Table 2.3). The amount of ammonium nitrogen in the water layer was almost the same at the end of the incubation period at the various dissolved oxygen concentrations used and amounted to 2.3 and 0.3 mg for sediments A and B, respectively. Nitrite nitrogen could not be detected in the overlying water.

The average denitrification rate (mg NO_3^--N $m^{-2} day^{-1}$) was calculated from the area of the sediment surface and the decline in the nitrate-nitrogen content of the overlying water during 260 hours of incubation (Table 2.3). The denitrification rate in sediment A decreased with an increased dissolved oxygen concentration in the water layer from 0 to about 3 mg l^{-1} (Fig. 2.7). Further increase of the dissolved oxygen concentration in the

Table 2.2. Some chemical data of sediments A and B used in the experiment on the influence of oxygen and nitrate (mg per g wet weight).

	Sediment	
	A	B
Dry weight	650	780
Organic matter determined as volatile solids	24.6	11.1
Ammonium nitrogen	0.78	0.05
Organic nitrogen	0.92	0.26
pH	7.60	7.18

Table 2.3. Denitrification rates of two water-sediment systems, consisting of sediments A and B, respectively, at different dissolved oxygen concentrations in the overlying water.

Percentage oxygen in gas mixture	Dissolved oxygen mg l^{-1}	mg NO_3^--N per tube at start	mg NO_3^--N present per tube after 260 hours of incubation		Denitrification mg NO_3^--N m^{-2} day^{-1}	
			Sed. A	Sed. B	Sed. A	Sed. B
0	0.0	5.00	1.84	2.72	161	116
2.5	1.0	5.00	2.00	2.89	153	108
5	2.1	5.00	2.18	3.04	144	100
10	4.1	5.00	3.10	3.00	97	102
15	6.2	5.00	2.34	3.14	136	95
21	8.7	5.00	2.34	3.02	136	101

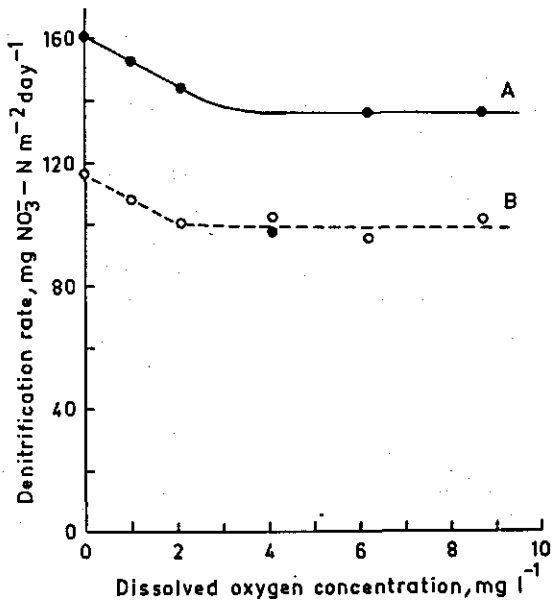

Fig. 2.7. The effect of the dissolved oxygen concentration in the overlying water on the denitrification rate in sediments A and B.

water layer had no further decreasing effect on the denitrification rate in the sediments. The denitrification rate in sediment B was lower than that in A at all oxygen levels, while the increase of the dissolved oxygen concentration had only a decreasing effect on the denitrification rate in the oxygen traject from 0 to 2 mg l^{-1} (Fig. 2.7).

The diffusion rates of nitrate and dissolved oxygen into the sediment are only depending on the concentration gradients, because the diffusion coefficients are almost equal, *viz.* 1.85×10^{-5} (Handbook of Chemistry and Physics, 1971–1972) and 2.6×10^{-5} cm^2 sec^{-1} (Kolthoff & Miller, 1941), respectively. From these data it may be concluded that oxygen as well as nitrate diffuse into the upper layer of the sediment, if the overlying water contains both components. In this layer of sediment oxygen is mainly used as electron acceptor by bacteria, because denitrification is inhibited by

oxygen (Wuhrmann & Mechsner, 1965; Chapter 9). Denitrification proceeds particularly in the lower sediment layers where no diffused oxygen is present. The thickness of the sediment layer into which oxygen penetrates presumably increases with the rise of the dissolved oxygen concentration in the overlying water. This results in an increased length of the supply route of nitrate to the sediment layer in which denitrification is possible. The decreasing denitrification rate with increasing dissolved oxygen concentration, at the lower oxygen levels (Fig. 2.7), might be the result of the increasing length of this supply route of nitrate. No explanation can be given for the constant denitrification rate in the sediments at higher dissolved oxygen concentrations in the overlying water.

The higher denitrification rate in sediment A compared with B was probably caused by the presence of a larger amount of available organic matter in sediment A (Table 2.2).

The influence of the nitrate-nitrogen concentration on the denitrification rate

In this experiment the sediments were taken from the same stock as in the last-mentioned experiment (Table 2.2). The water-sediment systems were incubated for only 3 days at 25°C to keep the decrease of the nitrate-nitrogen concentration during this period rather small, but at the same time the disappearance of nitrate nitrogen correctly measurable. After incubation nitrite nitrogen was not detectable in the overlying water, while the ammonium-nitrogen concentrations were broadly the same at all nitrate levels, *viz.* 27.0 and 0.0 mg l^{-1} ammonium nitrogen for sediments A and B, respectively. The losses of nitrate during the 5 periods of three days of incubation were equal.

The average denitrification rate was computed from the decrease of nitrate nitrogen in the overlying water during three days of incubation and the area of the sediment surface (Fig. 2.8). The denitrification rates in both sediment types were dependent on the nitrate concentrations in the overlying water, at lower concentrations closely approximating first

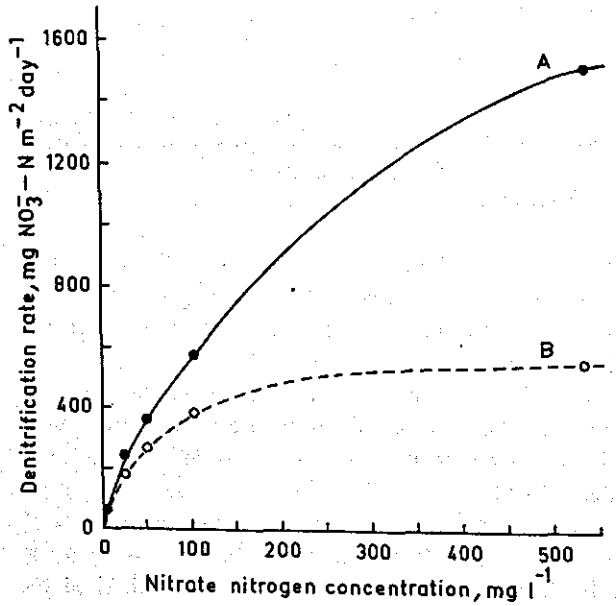

Fig. 2.8. The influence of the nitrate concentration in the overlying water on the denitrification rate in sediments A and B.

order kinetics and gradually becoming independent of the nitrate concentration at higher nitrate contents (zero order kinetics). With sediment B the maximum denitrification rate was reached at about 300 mg l^{-1} nitrate nitrogen, while that of A was not reached within the range of nitrate concentrations used (0–500 mg l^{-1} nitrate nitrogen). Empirically, the denitrification rates in both types of sediment approximated the Michaelis-Menten kinetics for nitrate concentrations, although the circumstances in the water-sediment system hardly complied with the terms of that theory. In a water-saturated soil enriched with glucose, denitrification rates were observed which also closely approximated Michaelis-Menten kinetics (Bowman & Focht, 1974).

The influence of the thickness of the layer of sediment on the denitrification rate

This experiment was carried out to obtain some information about the thickness of the layer of sediment which was involved in denitrification. Some chemical data concerning sediments A and B used for this experiment are presented in Table 2.4.

The decline of the nitrate-nitrogen concentration during the incubation period of 4 days was almost proportional to the time. The denitrification rate in mg NO_3^--N $m^{-2} day^{-1}$ was calculated from the area of the sediment surface and the nitrate consumption during incubation and it was plotted versus the corresponding layer thickness (Fig. 2.9).

The denitrification rates in sediments A and B were dependent on the layer thickness up to circa 7 and 14 mm, respectively. This means that at a nitrate-nitrogen concentration in the overlying water of 25.2 mg l^{-1} at the beginning of the experiment, a 7 and 14 mm thick layer of sediment type A and B, respectively, seemed to be involved in denitrification. So at this concentration in the overlying water and under the used experimental conditions, nitrate transport, which was mainly the result of diffusion, occurred for a distance of at most 7 and 14 mm into sediments A and B, respectively.

Transport of nitrate

The exchange of nitrate between water and sediment was of great importance in this study. The occurrence of denitrification in the sediment required transport of nitrate from the overlying water into the sediment. The experiments were arranged in such a way

Table 2.4. Some chemical data of sediments A and B used in the experiments concerning the influence of the thickness of the layer of sediment (mg per g wet weight).

	Sediment	
	A	B
Dry weight	740	725
Organic matter determined as volatile solids	20.1	11.6
Ammonium nitrogen	0.06	0.04
Organic nitrogen	0.41	0.33
pH	7.80	7.41

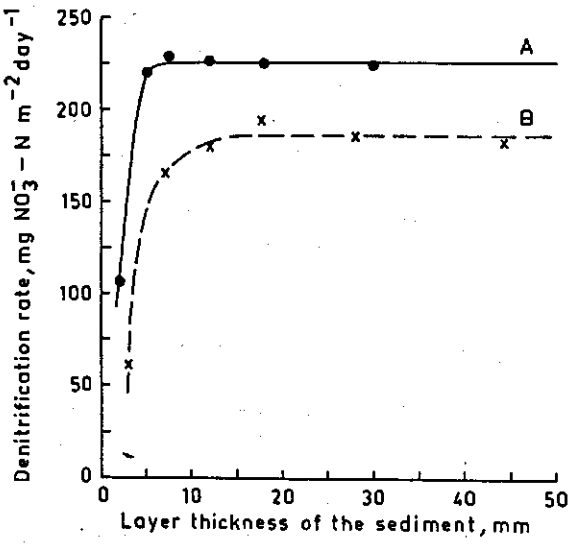

Fig. 2.9. The influence of the thickness of the layer of sediment on the denitrification rate in sediments A and B.

that there was no mass flow of water into the sediment. Therefore, diffusion was the driving force of the movement of nitrate from the overlying water into the sediment (Beek & Frissel, 1973). Diffusion depends on the concentration gradient and the diffusion coefficient. The latter is lower in the sediment than in the overlying water, because, firstly, the volume of sediment is only partly occupied by water and secondly, the water-filled pores in the sediment form no straight capillaries into the vertical direction. These capillaries form a labyrinth through which the diffusion path between two points is longer than the direct distance.

Molecular diffusion is not the only possible mechanism controlling the transport of nitrate through the water-sediment interface. In a review about factors affecting the transfer of materials between water and sediments, Lee (1970) considered also non-diffusional mechanisms of transport. The main non-diffusional mechanisms for the transport of nitrate seem to be those which cause mixing in sediments. This involves mechanical displacement of the interstitial water from its original location which allows a direct exchange between interstitial water and overlying water. Mixing in sediments may result from firstly, the movement of water directly above the water-sediment interface, secondly, the release of carbon dioxide and methane gas bubbles, produced by anaerobic fermentation reactions of bacteria in sediments and thirdly, by the continual re-working of sediments by bottom organisms (Rhoads, 1963) and the action of benthic fauna on and in sediments. Mixing in sediments is generally limited to the top few millimeters or centimeters, depending upon the energy of mixing and the type of sediment present (Paddock, 1975). It will be obvious that in the previous experiments the contribution of non-diffusional transport mechanisms to the transport of nitrate into the sediment was much less than in nature. Therefore, the denitrification rates in sediments under natural conditions will be higher than those found in the present investigation.

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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.

Introduction

In a previous investigation it has been demonstrated that nitrate may disappear from the surface water as a result of the interaction of the water and the sediment (Chapter 2). The nitrate diffuses into the sediment and is used as H-acceptor by the denitrifying bacteria, resulting in the conversion of nitrate into molecular nitrogen. This process is considered by the author as a natural and consequently as a cheap method to remove nitrate from shallow surface waters.

The microorganisms involved in denitrification utilize a certain amount of nitrogen for the synthesis of cellular material (immobilization). The immobilized nitrogen may originate from organic, ammonium or nitrate nitrogen.

In the present study it was investigated which part of the immobilized nitrogen originates from nitrate and which part from other sources.

To quantify the above-mentioned processes, experiments have been performed on a laboratory scale. Nitrate was added to a system consisting of a sediment layer and a water layer which was incubated in the dark and under anaerobic conditions. The nitrogen transformations occurring in such a system are mainly a result of denitrification, immobilization and ammonification. The immobilization of nitrogen is limited to heterotrophic and autotrophic denitrifying bacteria and to facultatively and strictly anaerobic microorganisms. The autotrophic organisms include bacteria which are able to oxidize sulphureous compounds with nitrate as H-acceptor (*e.g. Thiobacillus denitrificans*). Heterotrophic bacteria requiring molecular oxygen as hydrogen acceptor and the autotrophic

nitrifying bacteria do not grow under such conditions.

The transformations of nitrogen occurring in the model system were studied by using labeled nitrogen.

Materials and methods

Sediments

The experiments were carried out with two types of sediments, A and B. Type A originated from the 10-cm upper layer of a ditch into which effluent of a purification plant is discharged. This plant, consisting of an activated sludge tank and a final settling tank, is only fed with slurry from calfs destinated for meat production. Type B originated from the 10-cm upper layer of a ditch into which water from arable land is drained off. Some data about these sediments are shown in Table 3.1.

Design of experiments

Twenty gram of homogenized wet sediment was added to each of a number of glass tubes (inner diameter 3.6 cm; total volume about 225 ml) and supplied with 20 ml of a solution containing different amounts of ammonium chloride or sodium nitrate as indicated in Table 3.2. In some cases the nitrogen of the added nitrogenous compounds was labeled and contained 96.6 and 95.0 atom % excess ^{15}N, respectively. After the addition of the 20 ml solution the oxygen was displaced by flushing with helium. When all of the oxygen had been driven out, the tubes were gastight closed with a rubber stopper and butylrubber paste and incubated in the dark at 25°C.

The tubes numbered 10 and 20 were used for the determination of the non-biological fixation of ammonium. Both tubes were shaken, kept at 25°C for 24 hours and stored at –20°C to prevent microbiological immobilization.

The contents of the remaining tubes were analysed when the added nitrate had disappeared from the water-sediment system. This was determined in two additional tubes which had received the same amount of unlabeled nitrate as was used in the tubes tested for immobilization of nitrogen.

Table 3.1. The composition of 20 g wet sediment.

	Sediment	
	A	B
Dry weight	14.00 g	15.36 g
Organic matter determined as volatile solids	0.47 g	0.21 g
Ammonium nitrogen	4.1 mg	0.29 mg
Organic nitrogen	24.7 mg	6.22 mg
pH[a]	7.85	7.08

[a] The pH was measured after diluting 20 g of wet sediment with 50 ml de-ionized water.

Table 3.2. Inorganic nitrogenous compounds present in 20 ml de-ionized water added to tubes containing 20 g of wet sediment A or B.

Tube no.	Type of sediment	NH_4^+-N (μmoles)		NO_3^--N (μmoles)	
		labeled[a]	unlabeled	labeled[b]	unlabeled
7,8,9,10	A	1322	–	–	–
4,5,6	A	1322	–	–	857
1,2,3	A	–	–	800	–
19,20	B	529	–	–	–
17,18	B	529	–	–	286
15,16	B	–	571	266	–
13,14	B	–	286	266	–
11,12	B	–	–	266	–

[a] Containing 96.9 atom % excess ^{15}N.
[b] Containing 95.0 atom % excess ^{15}N.

Estimation of ammonium nitrogen

At the end of the experiment 0.1 ml of 10 N HCl was added to the contents of the tubes with a syringe to convert possibly occurring ammonia into ammonium ions. After one day the sediment and the water of each tube were quantitatively transferred to a 500-ml Kjeldahl flask followed by the addition of 50 ml borate buffer (pH 8.6) containing 8 g of H_3BO_3 and 40 g of $Na_2B_4O_7.10H_2O$ per litre. The ammonia was steam-distilled and collected in 15 ml of a 4% boric-acid solution followed by titration with 0.02 N H_2SO_4 using methylredbromocreosolgreen as indicator.

In a preliminary experiment it was investigated whether part of the organic nitrogenous compounds might be hydrolysed to ammonia during the steam-distillation with borate buffer, resulting in an overestimation of ammonia. This was tested with peptone (Bacteriological Peptone L 37 of Oxoid) in the absence and presence of sediment. From the results obtained it was concluded that no significant part of the organic nitrogen was hydrolysed to ammonia during the distillation.

Estimation of organic nitrogen

Organic nitrogen was estimated in the same flasks after the removal of the ammonia. The contents of the flasks were acidified, the water volume reduced by evaporation and the organic material digested according to Bremner (1965). After digestion and addition of an excessive amount of NaOH, the ammonia was steam-distilled and collected in 15 ml of a 4% boric-acid solution followed by titration with 0.02 N H_2SO_4.

Estimation of isotopic composition

In the case of samples containing ^{15}N, the ammonium obtained in the various distillations was distilled off again and collected in 0.1 N HCl. The ^{15}N estimations were performed according to Faust (1967) partially modified by Akkermans (1971). The

emission spectra of $^{14}N_2$, $^{14}N^{15}N$ and $^{15}N_2$ were obtained with a ^{15}N-analyser Straton NOI–4 after conversion of the ammonium N into N_2 and evaluated according to the method described by Ferraris & Proksch (1972).

Results and discussion

The nitrate added to the tubes with sediment A and sediment B had been consumed after 42 and 25 days of incubation, respectively. The immobilization of labeled ammonium during the incubation periods was found to be increased by the presence of nitrate, which has functioned as hydrogen acceptor (Table 3.3). Most of the added nitrate was lost by volatilization due to denitrification, *viz.* 97.2% in sediment A and 94.5% in sediment B (Table 3.3). From these results it can be concluded, that the disappearance of nitrate from the surface water by the interaction of the water and the sediment was almost complete.

From the remaining part of the added nitrate in sediment A, 62% was converted into ammonia and 38% was immobilized in organic matter. With sediment B these figures amounted to 36 and 64%, respectively. The latter values correspond with those obtained by Chen *et al.* (1972) in sediment from Lake Mendota. The ammonium nitrogen found to be derived from nitrate may have been formed by denitrification, and by ammonification of the previously immobilized nitrate. Ammonification of the previously immobilized nitrate would be of more importance with increased incubation time. This would explain the fact that in sediment A which had been incubated for a considerably longer period than B considerably more nitrate had been transferred to NH_4^+ than to organic nitrogen. Formation of ammonia from nitrate by denitrifying bacteria may under certain conditions be carried out by bacteria of the *Bacillus licheniformis* type (Verhoeven, 1952; Woldendorp, 1963).

Table 3.3 contains only the transformations of compounds containing ^{15}N. A more complete picture of the transformations is obtained when the ^{14}N of the inorganic

Table 3.3. Immobilization of labeled nitrate and ammonium nitrogen (μmoles/tube), and the effect of added nitrate on the immobilization of NH_4^+-^{15}N in sediments A and B.

Tube no.	Incubation time days	Initial amount of inorganic nitrogen				Distribution of ^{15}N after incubation			
		NH_4^+		NO_3^-		NH_4^+ derived from		Organic + fixed N derived from	
		^{15}N	^{14}N	^{15}N	^{14}N	NH_4^+-^{15}N	NO_3^--^{15}N	NH_4^+-^{15}N	NO_3^--^{15}N
Sediment A									
10	1	1277	338	–	–	1261	–	14	–
7,8,9	42	1277	338	–	–	1243	–	30	–
4,5,6	42	1277	338	–	857	1180	–	76	–
1,2,3	42	–	293	760	40	–	13	–	8
Sediment B									
20	1	511	39	–	–	499	–	3	–
19	25	511	39	–	–	499	–	10	–
17,18	25	511	39	–	286	485	–	20	–
11,12	25	–	21	254	12	–	5	–	9

compounds present at the beginning of the experiments is included in the calculations (Table 3.4). The ammonium originating from nitrate is considered in this table and in the following paragraphs as nitrogen resulting from ammonification of previously immobilized nitrogen and is therefore included in the fraction indicated as 'Fixed + immobilized N'.

The figures obtained for the various treatments (Table 3.4) enable to differentiate between the immobilization caused by strictly anaerobic processes (fermentation) and the immobilization caused by the denitrifying bacteria (nitrate respiration) in the sediments containing nitrate. Moreover, the figures of Table 3.4 allow to calculate which part of the immobilized nitrogen originates from the ammonium pool and which part originates from the nitrate nitrogen. The immobilized nitrogen in the presence of nitrate amounted to 100 and 34 μmoles per tube with sediments A and B, respectively, of which 20 and 8 μmoles per tube can be ascribed to immobilization due to fermentation and 80 and 26 μmoles per tube, respectively, to immobilization due to denitrification (Table 3.4). In this calculation it is assumed that the anaerobic processes proceeding in the sediments in the absence of nitrate are not affected when nitrate is added. This may not entirely be true so that as a consequence the calculated amount of immobilized nitrogen due to the denitrifying organisms (obtained by subtracting the values for immobilized nitrogen in the absence of nitrate from those in the presence of nitrate) may be somewhat too low. In both sediments the calculated quantity of nitrogen immobilized upon the addition of nitrate amounted to 10% if expressed as percentage of the amount of nitrate nitrogen added to the tubes. This figure corresponds with the theoretical value if it is assumed that in denitrification 70% of the carbon compound (H-donor) is respired and 30% is transformed to cell material. Such a distribution may occur in denitrification brought about by bacteria of the *Pseudomonas aeruginosa* type which are able to perform a

Table 3.4. Immobilization of nitrate and ammonium nitrogen, and non-biological fixation of ammonium nitrogen in sediments A and B (μmoles/tube).

Tube no.	Incubation time days	Initial amount of inorganic nitrogen		Fixed+immobilized N derived from			Fixed	Immobilized
		NH_4^+-N	NO_3^--N	NH_4^+-N	NO_3^--N[a]	Total		
Sediment A								
10	1	1615	–	18	–	18	18	–
7,8,9	42	1615	–	38	–	38	18	20
4,5,6	42	1615	857	96	22[b]	118	18	100
1,2,3	42	293	800	96[c]	22	118	18	100
Sediment B								
20	1	550	–	3	–	3	3	–
19	25	550	–	11	–	11	3	8
17,18	25	550	286	22	15[b]	37	3	34
11,12	25	21	266	22[c]	15	37	3	34

[a] The ammonium nitrogen originating from nitrate (see Table 3.3) is included in this fraction.
[b] These figures were derived from tubes 1,2,3 and 11,12, respectively.
[c] These figures were derived from tubes 4,5,6 and 17,18, respectively.

Table 3.5. The effect of different ammonium concentrations on the immobilization of nitrate N during 25 days of anaerobic incubation with nitrate as H-acceptor (μmoles/tube).

Initial amount of inorganic nitrogen		Distribution of labeled N after incubation		
NO_3^--^{15}N	NH_4^+-^{14}N	NH_4^+-^{15}N	Organic-^{15}N	Total-^{15}N
254	21	5	9	14
254	307	9	6	15
254	593	10	6	16

complete oxidation of the carbon compounds to H_2O and CO_2 under anaerobic conditions in the presence of nitrate.

As may be seen in Table 3.4, the nitrogen immobilized in sediment A as a result of denitrification originated only for about 30% from added nitrate N but for 70% from the ammonia pool of the sediment. This means that in this sediment only 2.8% of the added nitrate N was immobilized. The remaining part, derived from the ammonia pool, equals a value of 7.3 if expressed as percentage of the amount of added nitrate N. In sediment B a considerably larger part of the immobilized nitrogen was derived from the added nitrate N (*viz.* 5.5%) whereas 4.1% (calculated as % of nitrate N added) was obtained from the ammonia pool. The relatively high percentage of immobilized nitrate nitrogen in sediment B as compared with sediment A was thought to be due to the low concentration of ammonium nitrogen in sediment B. To check this hypothesis a number of tubes containing sediment B and added NO_3^--^{15}N, and supplied with different amounts of NH_4^+-^{14}N had been included in the above-mentioned experiment (Table 3.5). The total amount of labeled nitrogen in the ammonium and organic nitrogen fractions after 25 days of anaerobic incubation was not significantly different at the three ammonium nitrogen levels. However, when considering both fractions separately, it will be seen that the amount of labeled ammonia increased with increased ammonium nitrogen supply, whereas that of organic nitrogen fraction decreased. The latter result is in agreement with the above-mentioned hypothesis that high amounts of ammonium nitrogen depress the assimilation of nitrate nitrogen. No explanation can be given for the opposite behaviour of the fraction NH_4^+-N.

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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.

Introduction

In a previous study it was shown that nitrate disappeared from the aerobic overlying water of a water-sediment system, because nitrate diffuses into the anaerobic sediment in which it can be utilized by denitrifying bacteria as an electron acceptor (Chapter 2). In sediments A and B, originated from a ditch into which effluent of a purification plant is discharged and from a ditch into which the carry-over of rain water from arable land is drained off, respectively, most of the added nitrate nitrogen was lost by volatilization due to denitrification during anaerobic incubation, *viz.* 97.2% in sediment type A and 94.5% in B (Chapter 3).

Payne (1973) mentioned many aerobic heterotrophic bacteria able to utilize nitrate as terminal electron acceptor under anaerobic conditions. Also the aerobic chemoautotrophic bacterium *Thiobacillus denitrificans* may use nitrate as terminal electron acceptor under anaerobic conditions (Aminuddin & Nicholas, 1973). This respiratory nitrate reduction can proceed to different levels, *viz.* nitrite, nitric oxide, nitrous oxide, molecular nitrogen or ammonium, depending on the type and growth conditions of the bacterium.

Under natural conditions the gas emanating from aquatic sediments contains besides molecular nitrogen much methane (Reeburg, 1969). Methane may be formed during anaerobic bacterial degradation of organic matter according to several pathways (Wolfe, 1971). Also H_2, CO_2 and H_2S may be produced during anaerobic degradation in aquatic sediments.

In this study the production of gases in the presence and absence of nitrate was quantitatively measured in two anaerobically incubated water-sediment systems. Simultaneously, the contents of inorganic nitrogenous compounds in the overlying water of the sediments were determined.

Materials and methods

Sediments

Two types of sediment, A and B, were used. Type A originated from a 10-cm upper layer of a ditch into which effluent of a purification plant is discharged. This plant consists of an activated sludge aeration tank and a final settling tank. The plant is only fed with slurry produced by calfs destinated for meat production. Type B originated from a 10-cm upper layer of a ditch into which the carry-over of rain water from arable land is drained off.

Design of the experiments

The experiments were carried out simultaneously in two glass apparatuses (Fig. 4.1)

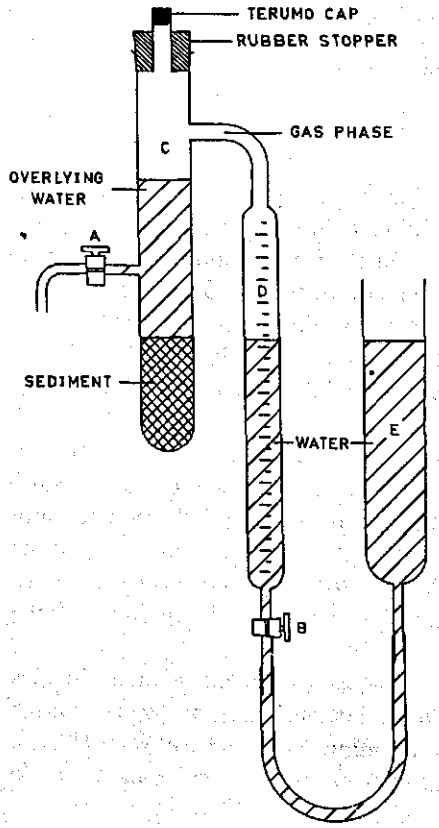

Fig. 4.1. Glass apparatus to measure the gas production at atmospheric pressure and to sample the gas phase and the overlying water of an anaerobically incubated water-sediment system.

The glass valves were slightly oiled with high vacuum grease. Tube D contained a scale graduated in millimeters to measure the volume of gas produced.

Tube C, having an inner diameter of 40 mm, was filled with 90 g of wet sediment. A perforated plate, having a diameter of 35 mm, was placed on the surface of the sediment enabling the addition of water without disturbing the surface of the sediment. After the addition of de-mineralized neutral water (200 ml to apparatus I and 215 ml to II) the perforated plates were carefully removed. Then the rubber stopper without the Terumo cap was tightly placed on the top of tube C using a permanently elastic paste on the basis of butyl-rubber to prevent leakages. After closing valve B, tube E was filled with de-mineralized water. During 20 minutes helium was bubbled through the water to make it free from air. After opening valve B and raising tube E this water filled tube D almost completely. Immediately thereafter the Terumo cap, stabbed by a thin needle, was placed on the glass tube. Subsequently the overlying water in tube C was flushed with helium (about 250 ml min^{-1}) via valve A for 10 hours. This manipulation did not disturb the surface of the sediment. Then the needle was removed and valve A closed. Apparatus I was supplied with 15 ml of a solution containing 50 mg of sodium nitrate nitrogen through the Terumo cap by means of a syringe. The sodium nitrate solution had been flushed with helium. A gas leakage possibly caused by the small holes left behind in the cap by the needle was prevented by smearing the upperside of the cap with a thin layer of high vacuum grease.

Atmospheric pressure inside the apparatus was obtained by opening valve B and moving tube E downwards until the water levels in tubes D and E were equal. Then valve B was immediately closed and the height of the water level in tube D noted whereupon the incubation at 25°C in the dark started. The overlying water and the gas phase were sampled weekly. As the pressure inside the apparatus exceeded the atmospheric pressure as a consequence of the production of gas, a sample of 5 ml overlying water could easily be taken by opening valve A. Thereafter, the atmospheric pressure in the apparatus was restored effecting a water stream from tube B to E. Consequently, no water with dissolved air was introduced into the system. The scale readings were noted and after a temporary removal of the high vacuum grease on the Terumo cap, gas samples of 100 µl were taken with a syringe.

The pH and the concentrations of nitrate, nitrite and ammonium nitrogen of the water sample were determined. The gas samples were analysed gaschromatographically weekly for He, N_2, NO, CH_4, CO_2 and N_2O and twice for H_2 and H_2S. The amount of produced gas was calculated from the differences in the scale readings. At the end of the incubation period, the total gas volume was determined by measuring the factor by which 100 µl ultra-pure ethane injected in the apparatus was diluted. The gas volume at each sampling time was computed from the final gas volume. Organic matter, ammonium and organic nitrogen contents of sediment B were determined at the end of the experiment. With both types of sediment the experiment was repeated once.

Gas-chromatographic analysis

For the determination of the He, H_2, N_2, NO, CH_4, CO_2, N_2O and H_2S contents of a gas sample the method described by Bailey & Beauchamp (1973) was slightly modified. A Becker type 406 Gas Chromatograph equipped with a thermal conductivity detector (TC)

with W2X filaments and a filament current of 250 mA was used. The temperature of the column oven was 50°C. A stainless steel column (900 cm X 2 mm) containing Porapak Q (80–100 mesh) was used. Hydrogen was used as carrier gas (flow rate of 25 ml min^{-1}) for measuring the concentration of He, N_2, NO, CH_4, CO_2, N_2O and H_2S. The hydrogen content was ascertained with helium as carrier gas.

Ethane was assayed with a Becker Multigraph type 409 Gas Chromatograph equipped with a flame-ionization detector. A 110 cm X 4 mm stainless steel column containing Porapak R with nitrogen as carrier gas was used. The temperature settings were: injection port 60°C, column oven 50°C and detector 120°C.

A gas sample of 100 µl was injected into the chromatograph.

Chemical analysis

The concentration of ammonium nitrogen in the overlying water was determined according to the Nessler method described in 'Deutsche Einheitsverfahren' (1960). Ammonium and organic nitrogen in the sediment were estimated according to Bremner (1965). The salicylate method was used for the estimation of nitrate nitrogen (Müller & Widemann, 1955), while nitrite nitrogen was determined with the reagent of Griess-Romijn van Eck described in NEN 3252 6.3 (1972).

Results

A 100 µl gas mixture containing low amounts of the gases under study was analysed according to the described gas chromatographic technique to show the improved separation and the retention times of the various gases (Fig. 4.2).

Some data about both types of sediment are shown in Table 4.1. Well homogenized wet sediment was used. The water content of sediment type A and B was 29.4 and 21.3%, respectively, based on wet weight.

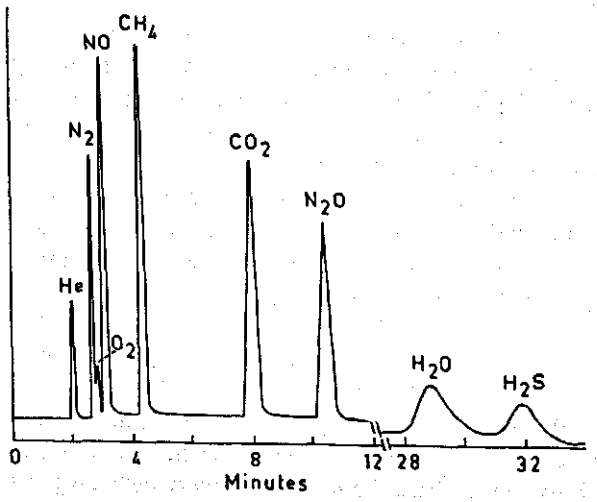

Fig. 4.2. Qualitative separation and retention times of a gas mixture containing low amounts of He, N_2, O_2, NO, CH_4, CO_2, N_2O, H_2O and H_2S in H_2.

Table 4.1. Some chemical data of sediment types A and B (mg per g dry weight).

	Sediment	
	A	B
Organic matter determined as volatile solids	34.0	9.3
Ammonium nitrogen	0.14	0.03
Organic nitrogen	1.63	0.17
pH[a]	7.45	7.10

[a] The pH was measured after diluting 10 g of wet sediment with 25 ml de-mineralized water.

Sediment type A

At the start of the experiment with sediment type A the gas phase of the system with and without nitrate contained besides He only 1.00 and 0.80% molecular nitrogen, respectively. During the 70 days of incubation at 25°C the pH of the overlying water varied between 7.30 and 7.59 in the presence of nitrate and between 6.90 and 7.45 in the absence of nitrate. In both cases the pH of the overlying water increased slowly up to half-way the incubation period and subsequently decreased slowly.

The results of the chemical analyses of the gas phase were plotted versus the incubation time (Fig. 4.3). In the presence as well as in the absence of nitrate no H_2 and H_2S were detected in the gas phase half-way and at the end of the incubation period. The added nitrate disappeared from the overlying water within 70 days at 25°C. The average denitrification rate computed from the nitrate consumption during the 70 days of incubation and the sediment area was 585 mg NO_3^--N m^{-2} day^{-1}. The addition of nitrate to the water-sediment system with sediment type A did not significantly influence the CO_2 and CH_4 production and the accumulation of ammonium nitrogen in the overlying water (Fig. 4.3). A substantially larger amount of molecular nitrogen was found in the system with nitrate than in the system without nitrate. At the end of the experiment all of the nitrate had been converted into molecular nitrogen.

In the system with nitrate small amounts of nitrite in the overlying water and nitrous oxide in the gas phase were observed during the first 30 and 42 days of incubation, respectively. Nitric oxide was not detectable in the gas phase of that system.

The colour of the sediment without nitrate kept black during the incubation period, whereas that in the upper layer of the sediment with nitrate turned grey-brown (Fig. 4.4).

Sediment type B

At the start of the experiment with sediment type B the gas phase of the system with and without nitrate contained besides He only 0.90 and 0.97% molecular nitrogen, respectively. The pH of the overlying water varied in a similar way as it did in the experiment with sediment type A, between 7.21 and 7.65 in the presence, and between 7.02 and 7.30 in the absence of nitrate. In both systems no H_2, H_2S and NO was detected during the

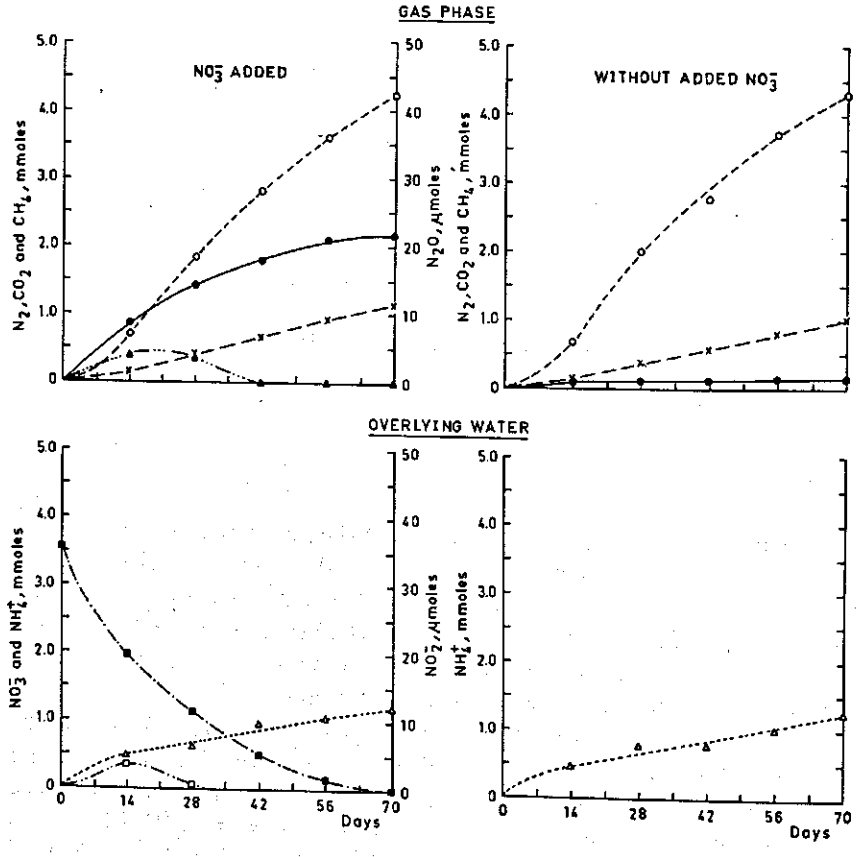

Fig. 4.3. The contents of N_2, N_2O, CH_4 and CO_2 in the gas phase and NO_3^-, NO_2^- and NH_4^+ nitrogen in the overlying water of a closed anaerobic water-sediment system containing sediment type A with and without added nitrate during incubation at 25°C. ●——● N_2; ▲–...–▲ N_2O; ○——○ CH_4; X– –X CO_2; ■–.–■ NO_3^--N; □..–..□ NO_2^--N; △– – –△ NH_4^+-N.

incubation period. The added nitrate did not disappear completely during 120 days of incubation (Fig. 4.5). The average denitrification rate was 221 mg NO_3^--N m^{-2} day^{-1}. In the system with nitrate, nitrite was present in the overlying water during the first 70 days, while nitrous oxide was detected in the gas phase during the first 100 days of incubation. With nitrate present more CO_2 but much less CH_4 was found than without nitrate. During the incubation the colour of the sediment kept black in the absence of nitrate and turned yellow-brown in the presence of this compound (Fig. 4.6). Some chemical data of both systems of sediment type B, ascertained at the end of the experiment, are listed in Table 4.2.

The experiments were repeated once with both types of sediments. The results obtained fully confirmed the trends of the recorded experiments. The results of both sets of experiments were not averaged as the samples of the first set of experiments were collected at a different date compared to those of the second set.

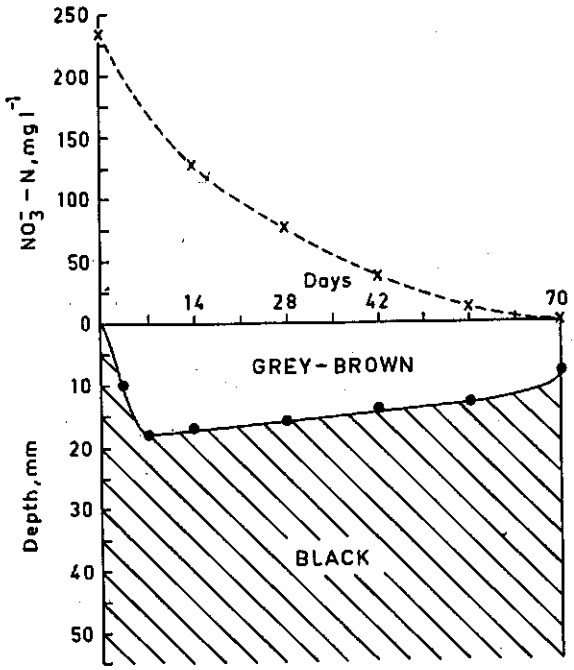

Fig. 4.4. The depth distribution of the grey-brown coloured sediment type A and the concentration of nitrate nitrogen in the overlying water during 70 days of incubation.
X– – –X Nitrate nitrogen in the overlying water.
●————● Border line of the grey-brown coloured sediment layer.

Discussion

The improved separation of He, N_2, O_2 and NO with the column used in this study (Fig. 4.2), compared with that described by Bailey & Beauchamp (1973), more than counterbalances the longer time needed for the analysis.

In the gas phase H_2S was not detected. This indicates that H_2S, certainly formed during the anaerobic decomposition of organic matter by strictly anaerobic bacteria, was probably precipitated at the pH value of the sediment.

The possibility of introducing errors into the estimation of the amounts of different gases due to differences in solubility of the gases in the overlying water must be taken into account, especially with nitrous oxide and carbon dioxide (Van Cleemput & Baert, 1972). Nitrous oxide is fairly soluble in water, but at the relatively low concentrations involved in the present study the amounts measured in the gas phase are about 25% below the amounts produced in the sediment. It is very difficult to discuss the carbon dioxide

Table 4.2. Some chemical data (mg per g dry weight) of sediment type B at the end of the experiment with and without nitrate.

	With NO_3^-	Without NO_3^-
Organic matter determined as volatile solids	7.8	8.3
Ammonium nitrogen	0.03	0.03
Organic nitrogen	0.26	0.13
pH	7.45	7.26

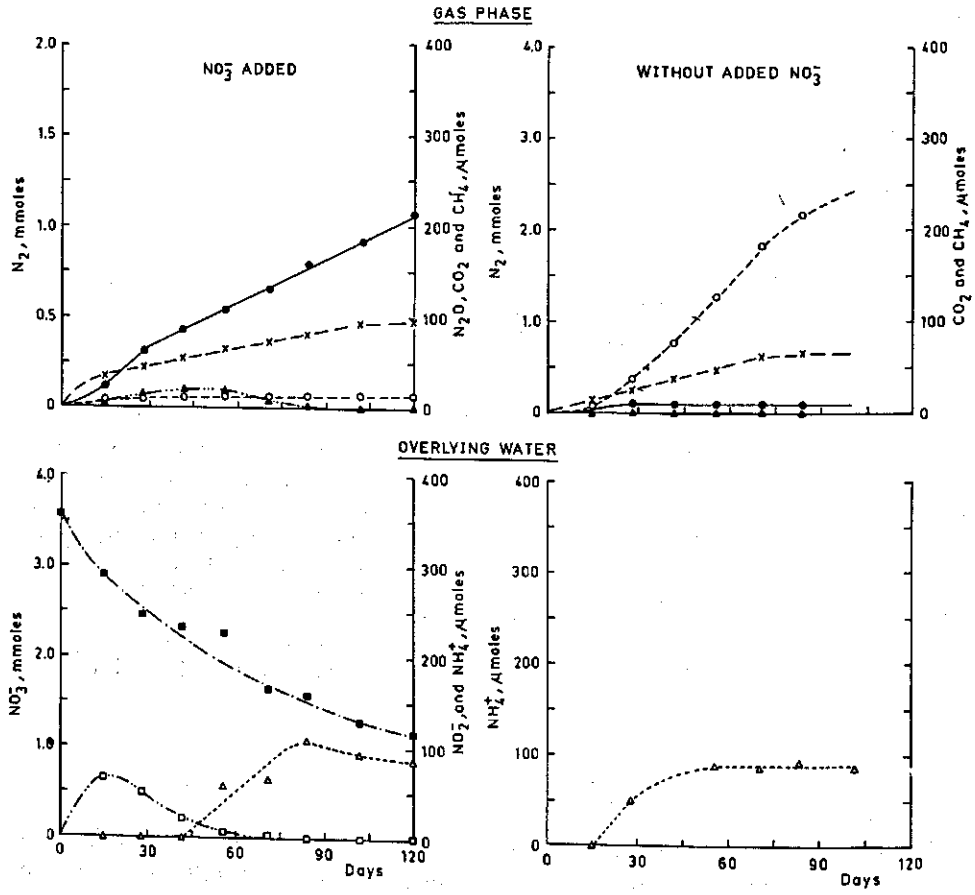

Fig. 4.5. The contents of N_2, N_2O, CH_4 and CO_2 in the gas phase and NO_3^-, NO_2^- and NH_4^+ nitrogen in the overlying water of a closed anaerobic water-sediment system containing sediment type B with and without added nitrate during incubation at 25°C. ●———● N_2, ▲–···–▲ N_2O; ○– – –○ CH_4; X— —X CO_2; ■ —·— ■ NO_3^--N; □··–··□ NO_2^--N; △- - - △ NH_4^+-N.

production, because on the one hand the rather high pH of the sediment may have resulted in precipitation of part of the carbon dioxide while on the other hand the formation of acids in microzones may have released carbon dioxide already present before the start of the experiments.

Nitric oxide is a well-established intermediate of the denitrification process (Walker & Nicholas, 1961). Payne & Riley (1969) stated that nitrate suppresses the activity of the nitric-oxide-reducing enzyme. This implies that NO would appear in the beginning of the incubation. However, no nitric oxide was detected in the gas phase by the gas-chromatographic method used. Therefore, the sequence of the different nitrogenous components, perceived during denitrification in aquatic sediments, appeared to be: $NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$. This sequence was also observed in soils (Cooper & Smith, 1963) and in sediment of Lake Mendota (Chen *et al.*, 1972). It was assumed that at the pH measured in the sediment, chemical decomposition of nitrite to gaseous products (see Broadbent &

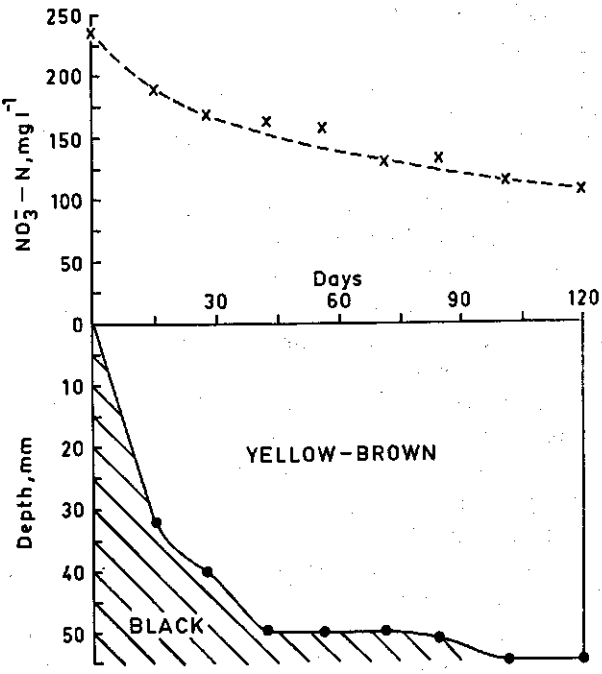

Fig. 4.6. The depth distribution of the yellow-brown coloured sediment type B and the concentration of nitrate nitrogen in the overlying water during 120 days of incubation. X– – –X Nitrate nitrogen in the overlying water. ●———● Border line of the yellow-brown coloured sediment layer.

Clark, 1965) was no significant.

Small amounts of nitrite in the overlying water and nitrous oxide in the gas phase were observed in both systems in the presence of nitrate in the beginning of the incubation period, when molecular nitrogen already accumulated in the gas phase (Figs. 4.3 and 4.5). This indicates that the reduction of nitrate to nitrite was faster than the reduction of nitrite to N_2O, while the latter reduction step was faster than that of N_2O to N_2. Upon prolonged incubation of both systems NO_2^- as well as N_2O disappeared, the former at an earlier date than the latter. An explanation of this phenomenon would be that owing to the decreased nitrate concentration in the overlying water, the nitrate diffusion into the sediment and as a consequence the nitrate supply of the denitrifying organisms decreased to such an extent that the rate of nitrate reduction dropped below the rate of nitrite reduction so that nitrite disappeared. Subsequently, the rate of nitrite reduction dropped below the rate of N_2O reduction which resulted in the disappearance of N_2O from the gas phase. No explanation can be given of the differences in the maximum amounts of nitrite and nitrous oxide between both types of sediment (Figs. 4.3 and 4.5).

In both systems without nitrate small amounts of molecular nitrogen accumulated in the gas phase during the incubation (Figs. 4.3 and 4.5). This molecular nitrogen had probably been pent up in the sediment before incubation and was released from the sediment together with carbon dioxide and methane. The difference in the denitrification rate occurring between the two types of sediment was related to the differences in content and availability of organic matter (Table 4.1).

The fading of both types of sediment in the presence of nitrate was a result of the oxidation of S^{2-} and Fe^{2+} (Figs. 4.4 and 4.6). The sulphide may have been oxidized to sulphate by *Thiobacillus denitrificans* using nitrate as electron acceptor under anaerobic

conditions (Aminuddin & Nicholas, 1973). Kuznetsov (1968) reported a number of bacteria that are able to oxidize ferrous to ferric ions. Sediment A faded from black into grey-brown and B from black into yellow-brown upon the incubation with nitrate, presumably because of the oxidation of the black coloured FeS. The grey-brown colour of the oxidized sediment A as contrasted to the yellow-brown sediment B was probably due to the much lower iron content of the former sediment.

From Fig. 4.4 it was concluded that the nitrate failed to diffuse deeper into the sediment A than about 2 cm. It is clear that in sediment A there was an abundance of available organic matter. In sediment B the depth distribution of the yellow-brown sediment increased continuously during the incubation (Fig. 4.6), because in this sediment the available organic matter was limited and nitrate remained present in excess.

Macgregor & Keeney (1973) reported that nitrate inhibited the formation of methane in sediments by raising the redox potential (Eh) of the sediment. Whether nitrate was present in the overlying water of the system with sediment A or not, the amount of methane formed in the sediment was equal (Fig. 4.3), because nitrate had prevented CH_4 formation only in the relatively narrow upper layer of the sediment (Fig. 4.4). In sediment B, however, much more methane was formed in the absence of nitrate than in the presence (Fig. 4.5). In the latter system the nitrate was present nearly throughout the sediment (Fig. 4.6), preventing CH_4 formation almost entirely.

The rates of methane formation per day per gram dry weight of sediments A and B without nitrate were 0.98 and 0.04 μmoles, respectively. The markedly higher CH_4 production in sediment A compared to B was due to the availability of much more organic matter usable for the formation of methane (Table 4.1).

Comparison of the organic matter contents of sediment B after incubation in the presence or absence of NO_3^- (Tables 4.1 and 4.2) suggests that during methane formation in the absence of nitrate less organic matter was decomposed than during denitrification. The low rate of denitrification in spite of the presence of organic material in sediment B at the end of the incubation period (Table 4.2 and Fig. 4.5) indicates that the major part of the organic matter of sediment B was not biodegradable and probably consisted of humic substances. During denitrification, which was the major process in sediment B supplied with nitrate, the organic nitrogen content of that sediment increased, possibly due to immobilization. However, in sediment B without added nitrate, in which methane formation was the major process, the organic nitrogen content decreased (Tables 4.1 and 4.2). This may have been the result of anaerobic decomposition of organic nitrogenous compounds.

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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.

Introduction

Proper evaluation of any biological transformation in an aquatic system requires consideration of the redox potential (Eh) within the system. The interpretation of the measured potentials have been discussed extensively by Stumm & Morgan (1970). The Eh of an aqueous system is relatively insensitive to variations of dissolved oxygen concentrations down to values of 0.1% saturation (Greenwood, 1962). After depletion of oxygen, some species of bacteria utilize alternative electron acceptors. It is generally accepted that nitrate is the first electron acceptor following depletion of oxygen. During this process of dissimilatory nitrate reduction (denitrification) nitrogen oxides and finally molecular nitrogen are produced. After depletion of nitrate, alternative electron acceptors such as MN^{4+}, Fe^{3+}, SO_4^{2-} and CO_2 may be utilized. The order with which these acceptors are used, is characterized by a declining Eh of the acceptors.

Mortimer (1941) used the redox potentials measured at various depths in lake sediments to estimate the penetration of oxygen into those sediments. Bailey & Beauchamp (1971, 1973) observed a stabilized Eh of + 200 mV in an anaerobic water-saturated soil in the presence of nitrate. Decomposition of organic matter by obligate anaerobic bacteria and the production of methane by methanogenic bacteria occur at Eh values below −150 mV (Ponnamperuma *et al.*, 1966). No nitrate is present at such low Eh values. Consequently, Eh values at various depths in sediments might be useful to estimate the position of the layer in which denitrification occurs.

In previous laboratory investigations (Chapters 2 and 4) an interaction between surface water and sediment was shown resulting in the removal of nitrate from surface water by the activity of denitrifying bacteria in the sediment.

The purpose of this laboratory study was to estimate the position of the denitrification zone in the sediment as a function of time. The available electron donors in this sediment became exhausted by maintaining a constant nitrate concentration in the overlying water.

Materials and methods

Sediment

The experiment was carried out with sediment from the upper 10-cm layer of the sediment of a ditch into which effluent of a purification plant is discharged. This plant consists of an aeration tank for activated sludge and a final settling tank. The plant is loaded with slurry from calfs destinated for meat production. The same type of sediment was already used in previous investigations (Chapters 2, 3 and 4).

Before use, the wet sediment was passed through a sieve with meshes of 2 mm to remove stones and subsequently well-homogenized. Some chemical data of the sediment are presented in Table 5.1.

Eh measurement

Eh measurements had to be carried out in the solution above the sediment and at various depths in a straight line down in the sediment. The use of several customary electrodes placed simultaneously at predetermined positions in the sediment was impracticable, so that a special probe was constructed (Fig. 5.1). This probe had a simpler construction than that developed by Machan & Ott (1972).

Each electrode consisted of a 10-mm long platinum wire with a diameter of 0.9 mm soldered with silver wire to one end of a shielded 1-conductor cable, of which the other end was connected with a Philips electrode plug. The six pieces of platinum wire were horizontally inserted into a teflon tube (O.D. 21.5 mm; I.D. 16 mm) at 0.5, 1.5, 2.5, 3.5, 4.5 and 8.5-cm distance from the bottom, respectively, and fixed with gum. The

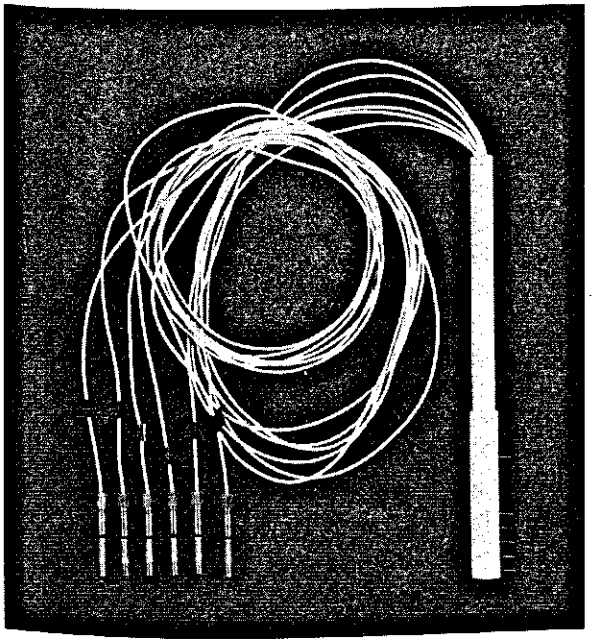

Fig. 5.1. The probe constructed for Eh measurements at various depths in a straight line down in a sediment.

Table 5.1. Some chemical data (mg per g dry weight) of the sediment before and after incubation of the water-sediment system with nitrate present.

	Before incubation	After incubation
Organic matter determined as volatile solids	30.0	15.8
Ammonium nitrogen	0.26	0.12
Organic nitrogen	1.10	0.63
pH	7.6	7.0

insulation resistance of the gum was above 10^9 ohms. Thereafter the opening at the bottom of the tube was sealed with a teflon stopper. Before use the platinum wires were cleaned with a mild scouring powder on a paper towel.

Measurements were made with a saturated calomel electrode as reference. The potential of the calomel electrode against a standard hydrogen electrode was added to the measured potential to calculate the Eh value. The measurements were carried out with an ion activity meter (Philips model PW 9413). The system was checked with a redox buffer of quinhydrone. No corrections were made for changes of the pH, because the pH of the overlying water was controlled at pH 7.0.

Experimental system

The experiment was carried out in a polymethyl metacrylate vessel (I.D. 19.0 cm and 20.0 cm high) with a working-volume of 5.5 l (Fig. 5.2). A 3-cm thick layer of sediment (934 g dry weight) was brought into the vessel. The area of the sediment surface was 283 cm^2. A 0.5-cm thick sheet of rough foam-plastic was led upon the sediment layer, which on its turn was kept in place by means of a perforated 0.2-cm thick sheet of steel. These sheets prevented disturbance of the sediment, while the transport of soluble components between the sediment and the overlying water was hardly affected. The Eh probe was stuck through a hole in both sheets to the bottom of the vessel. Three platinum wires were lying in the sediment at 0.5, 1.5 and 2.5 cm, respectively, and the other three in the overlying water. A combined pH-glass electrode was put into the sediment through another hole in both sheets to measure the pH in the sediment at a depth of 1.5 cm. Then 3.5 l of an aqueous solution containing 5 mg l^{-1} N-Serve was put into the vessel and subsequently a cover placed on the vessel. For a complete inhibition of the nitrification process 17.5 mg N-Serve was added every two weeks (Chapter 2). The cover contained besides holes for the Eh and pH probes another 10 holes which were used for other purposes. The dissolved oxygen concentration of the overlying water was maintained at a high level by bubbling water-saturated air through the overlying water. The dissolved oxygen concentration was measured continuously with a polarographic sensor connected with an oxygen meter (WTW type OXI 39). A pH-glass electrode in the solution in combination with a pH meter (Radiometer type 28) and titrator (Radiometer type TTT 11b) was used to control the pH of the overlying water at 7.0 ± 0.1. The burette of the titrator was filled with 1 N HCl. A nitrate-ion electrode (Orion model 92–07) connected with a specific ion activity meter (Radiometer type 26c) was used for continuous measurement of the change in nitrate concentration. This system in combina-

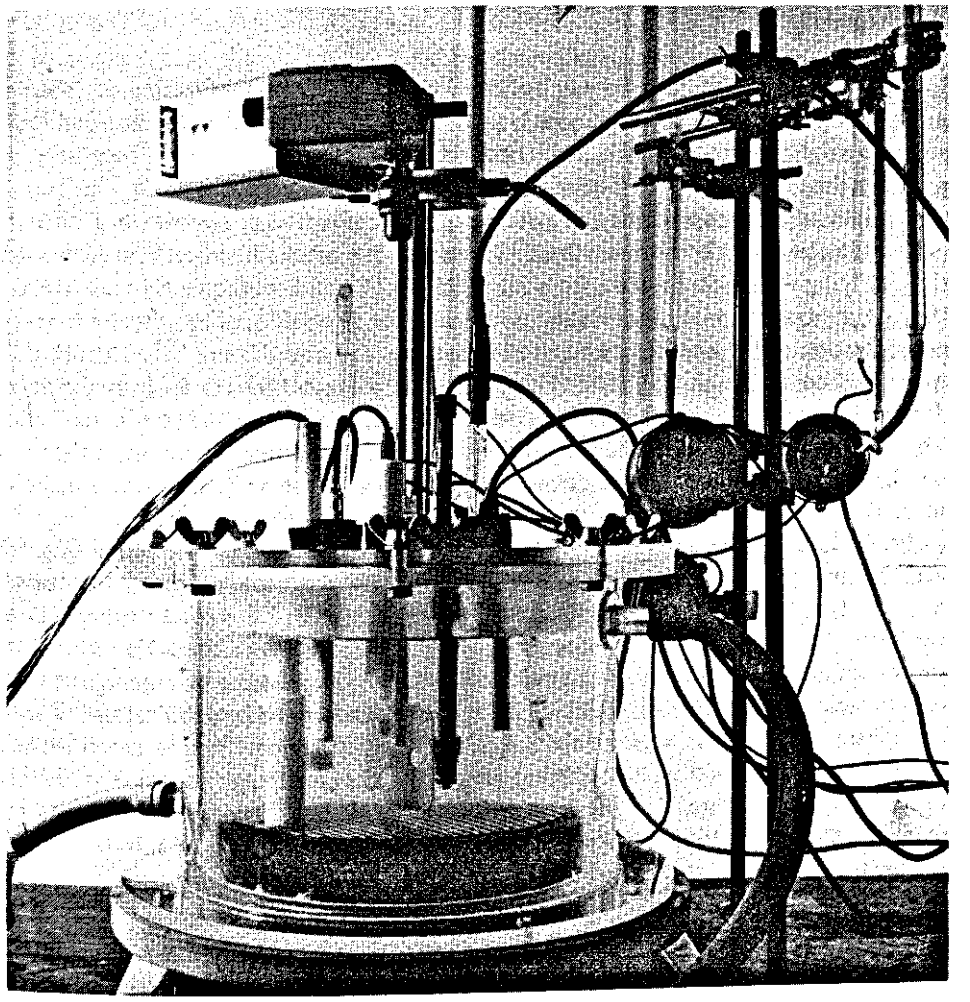

Fig. 5.2. The experimental system used.

tion with a titrator (Radiometer type TTT 11b) and a burette filled with a solution containing 2500 mg l^{-1} sodium nitrate nitrogen, was used to maintain the concentration of nitrate nitrogen in the overlying water at 25 mg l^{-1}.

All of the electrodes except the oxygen sensor and the combined pH-glass electrode were used in combination with a double junction reference electrode (Philips type R44/2-SD/1). The outer chambers of all the reference electrodes used were filled with 0.1 M NaCl.

A stirring-rod with a large rudder was used for mixing the overlying water (stirring-speed about 60 r.p.m.). The temperature in the vessel was controlled at 15°C by means of a water-jacket.

All measuring systems were checked weekly and if necessary calibrated again. The concentration of nitrate, nitrite and ammonium nitrogen in the overlying water were determined chemically, also weekly (Chapter 2).

Results and discussion

The experiment was carried out in the dark to avoid immobilization of inorganic nitrogenous compounds by phytoplankton. Nitrification also did not occur due to the addition of N-Serve. An incubation temperature of 15°C was chosen because it approaches the temperature often occurring in sediments of natural waters.

Actually, the experiment started after an incubation period of two weeks. During this period the electrodes became stabilized. Thereafter, the nitrate-nitrogen concentration in the overlying water was brought from nil to 25 mg l^{-1} and subsequently maintained at that concentration with a deviation of less than 1 mg l^{-1}. The dissolved oxygen concentration of the overlying water varied between 6.1 and 9.0 mg l^{-1}. The pH of the water was controlled at 7.0 ± 0.1 The pH in the sediment decreased from 7.6 to 7.0 during the stabilizing period but remained constant during the further incubation period. The overlying water was clear during the 241 days of incubation as a result of the sheets on the surface of the sediment. Some chemical data of the sediment before and after incubation with nitrate are presented in Table 5.1.

From the amount of nitrate disappeared during the 241 days of incubation (Fig. 5.3) and the surface area of the sediment (0.0283 m^2), the average rate of nitrate removal was calculated and was found to amount to 160 mg NO_3^--N m^{-2} day^{-1}. In a previous investigation with the same type of sediment (Chapter 3) 97.2% of the added nitrate was shown to be converted to gaseous products (denitrified) whilst the remaining part of the nitrate was used for the synthesis of bacterial cell material (immobilization). So, the disappearance of nitrate was mainly a result of denitrification. In this experiment the denitrification occurred in the sediment and not in the overlying water due to the high dissolved oxygen content of the latter which strongly impaired denitrification (Wuhrmann & Mechsner, 1965). Therefore, prior to the disappearance of nitrate from the system the nitrate had to diffuse into the sediment.

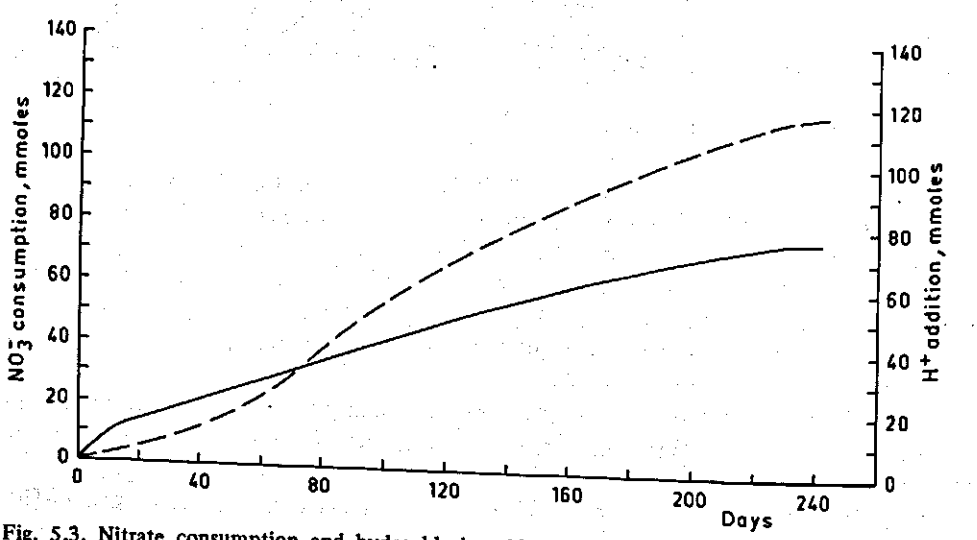

Fig. 5.3. Nitrate consumption and hydrochloric acid used for neutralization during the period of incubation. ——— NO_3^- and – – – H^+.

In addition to nitrate also oxygen diffused into the sediment. Oxygen respiration occurred mainly in the upper layer, while denitrification proceeded particularly in a deeper layer where no oxygen was present. When the available electron donors in the upper layer became exhausted, oxygen diffused into a deeper layer where electron donors were still available. This resulted into a shift of the zone of denitrification to a deeper layer. The course of this shift with time was followed indirectly by measuring the Eh in the sediment at three depths (Fig. 5.4). In the following paragraph it is shown that the depth at which denitrification occurred could be derived from the Eh profile in the sediment.

The measured redox potentials were mixed potentials which can not be considered as conceptually defined oxidation-reduction potentials, but they were useful in monitoring the extension of the oxidized part of the sediment. The Eh of the aerated overlying water varied between +315 and +450 mV (Fig. 5.4). At the start of the experiment the Eh values in the sediment layer at a depth of 0.5, 1.5 and 2.5 cm were –220, –225 and –160 mV, respectively, indicating highly reduced conditions. The Eh at a depth of 0.5 cm rose to +310 mV in 70 days. At 1.5 cm the Eh was maintained at about –225 mV during the first 80 days, whereafter it rose to about + 325 mV during the next 80 days. The Eh at a depth of 2.5 cm remained about –160 mV during 150 days and rose thereafter in 35 days to + 125 mV. It is assumed that if nitrate diffuses into a highly reduced layer of sediment, the Eh starts to rise until at most + 100 mV under denitrifying conditions and that more positive values are attained only if oxygen diffuses into that layer. These assumptions are based upon the observation of Bailey & Beauchamp (1971) who found that the Eh of an anaerobic water-saturated soil supplied with nitrate or nitrite reached a value of +200 mV and upon the statement of Hargrave (1972) who showed that at an Eh below

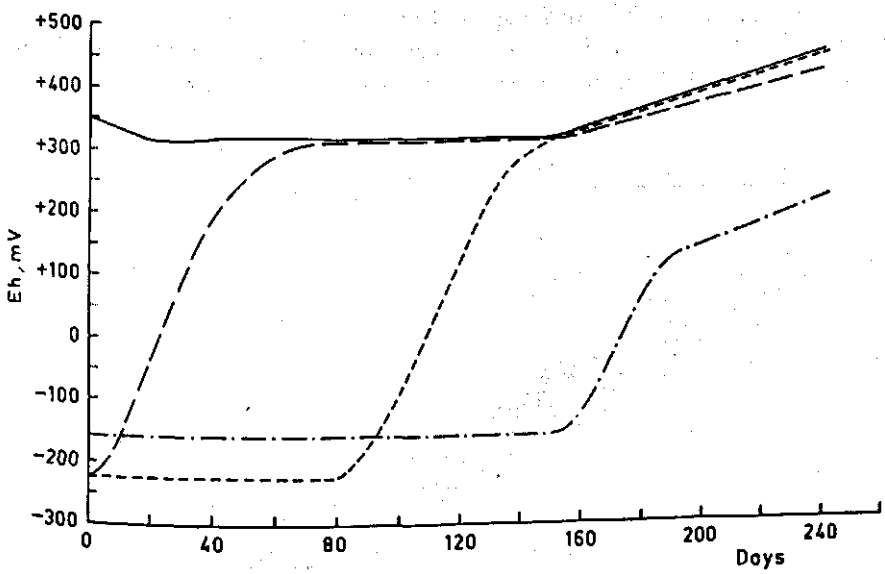

Fig. 5.4. Eh values of the overlying water and of the sediment at 3 depths during the period of incubation. ——— Overlying water; – – – 0.5, - - - - 1.5 and –.–.– 2.5 cm depth in the sediment.

+ 100 mV no oxygen can be detected in a sediment. The course of the front of nitrate in the sediment of the present experiment was figured out by taking the points of time at which the Eh of the highly reduced sediment started to rise at the three positions in the sediment. The same was done for oxygen by taking the points of time at which the Eh became higher than + 100 mV at those three positions (Fig. 5.5). From these results the zone in which denitrification occurred was derived. The course of this zone was followed during the incubation period. It is concluded, that oxygen reached the bottom of the vessel at about the 235th day of incubation, which is in agreement with the observation that nitrate was no longer consumed for dissimilatory purposes after that time (Fig. 5.3). The Eh value in the sediment at a depth of 2.5 cm did not rise in the same way as at a depth of 0.5 and 1.5 cm (Fig. 5.4). This may have been caused by poisoning of the Pt-electrode after exposure to −160 mV for a prolonged time (Bailey & Beauchamp, 1971). However, Graetz *et al.* (1973) found no indication that poisoning of the electrode occurred in sediments of Lake Mendota.

At the beginning of the experiment only the upper 1-mm layer of the sediment was greyish brown whereas the rest of the sediment was black. During the incubation under nitrate-containing water also deeper layers of sediment turned greyish brown and after 235 days the whole sediment had taken that colour. The black colour of the highly reduced sediment (Fig. 5.4) was probably due to the presence of FeS. In the greyish brown sediment the iron was presumably in the oxidized state. In a previous investigation (Chapter 4) it was shown that the fading of sediment occurred under anaerobic conditions with nitrate present. It is obvious that in the present investigation the fading of the sediment also occurred mainly under these circumstances as nitrate diffused more deeply into the sediment than oxygen (Fig. 5.5). Kuznetsov (1968) reported the oxidation of ferrous to ferric ions by a number of species of bacteria, while S^{2-} might be oxidized to sulphate by *Thiobacillus denitrificans* with nitrate as electron acceptor (Aminuddin & Nicholas, 1973) and by other *Thiobacillus* sp. in the presence of oxygen.

In the beginning of the incubation period small amounts of nitrite were detected in the overlying water (Fig. 5.6). As nitrification was inhibited, this nitrite must have originated

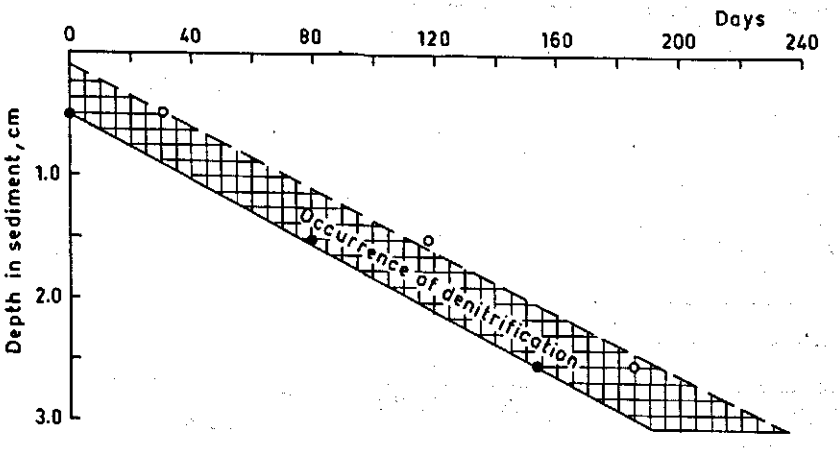

Fig. 5.5. The shift of the denitrification zone during the period of incubation deduced from the depths of penetration of oxygen and nitrate. –o– Penetration of oxygen and –•– penetration of nitrate.

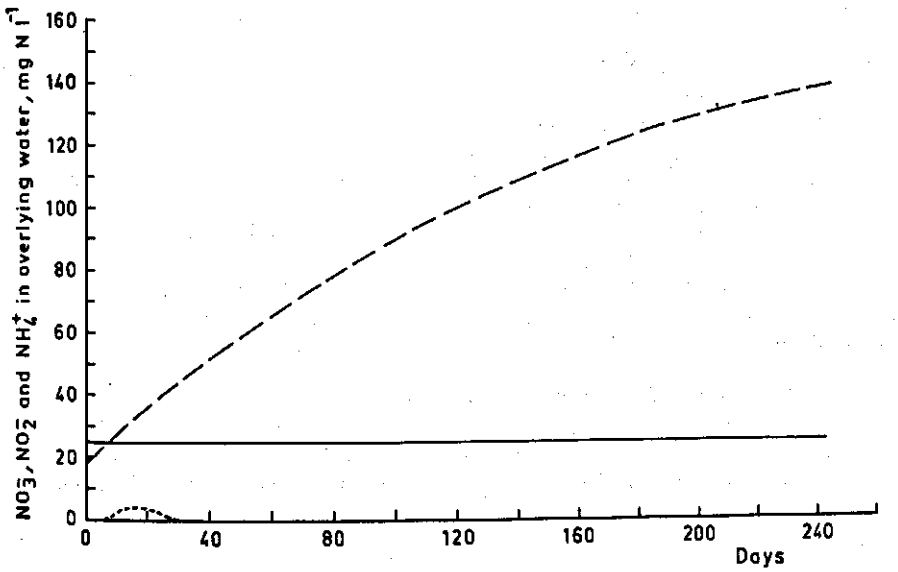

Fig. 5.6. Nitrate, nitrite and ammonium nitrogen in the overlying water during the incubation period. ——— NO_3^-, ----- NO_2^- and – – – NH_4^+.

from denitrification, a conclusion which is in accordance with previously obtained results (Chapter 3).

During the transformation of two moles of nitrate to one mole of molecular nitrogen, two moles of hydroxide are formed. In the beginning of the incubation period, part of the OH^- formed was buffered by the sediment (Fig. 5.3). In total more acid was added to the overlying water than was required for neutralization of the OH^- produced as a result of denitrification. This was caused by the release of ammonia from the sediment (Fig. 5.6). During denitrification nitrogen-containing organic compounds were decomposed, which gave rise to the formation of ammonia. Neutralization of the biologically formed ammonia also required addition of acid. At the end of the experiment about the same amount of acid had been added as was calculated from the amount of nitrate disappeared and ammonia formed.

After 230 days of incubation no further nitrate consumption occurred, because the hydrogen donors required for the denitrification process in the sediment were exhausted. From Table 5.1 it may be concluded that 53% of the organic matter present in the sediment was not used as hydrogen donor in denitrification during the experimental period, probably because it consisted of humic substances which are resistant to a ready microbial attack.

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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

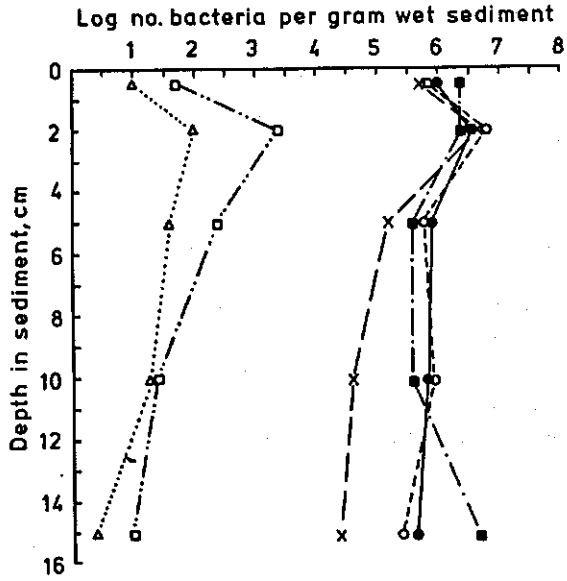

Fig. 6.1. Numbers of aerobic and anaerobic heterotrophic bacteria, and of denitrifying, ammonifying and nitrifying bacteria in a sediment core (August, 1975). ●———● Aerobic heterotrophs; ○- - -○ Anaerobic heterotrophs; X— —X Heterotrophic denitrifiers; ■——.——■ Ammonifying bacteria; □–..–□ *Nitrosomonas*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

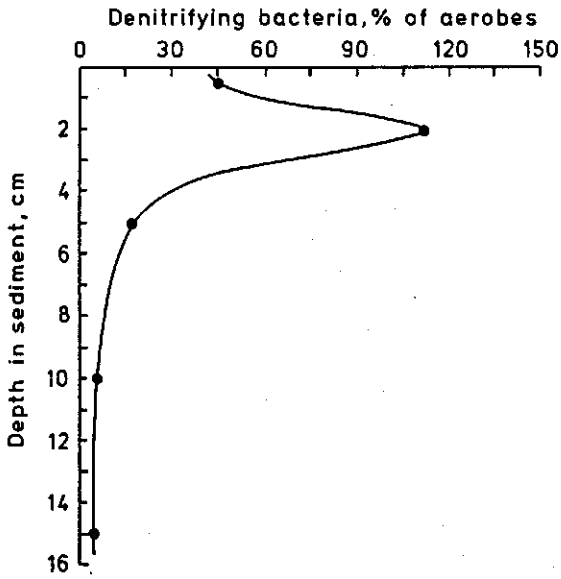

Fig. 6.2. Numbers of heterotrophic denitrifying bacteria as percentage of the numbers of aerobic heterotrophs in relation to depth in the sediment.

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

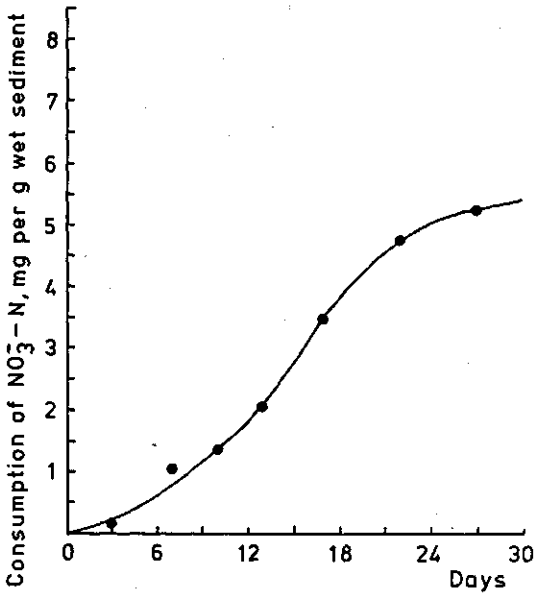

Fig. 6.3. Amount of nitrate nitrogen consumed by 1 g of wet sediment during incubation at 15°C.

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

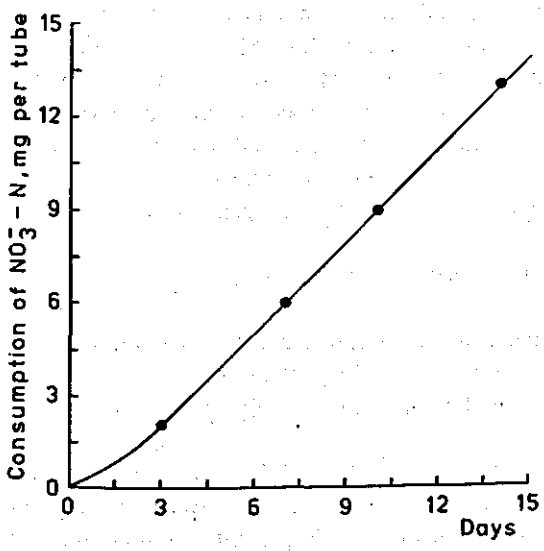

Fig. 7.1. Nitrate nitrogen consumed by the sediment of Experiment 1 during anaerobic incubation at 25°C.

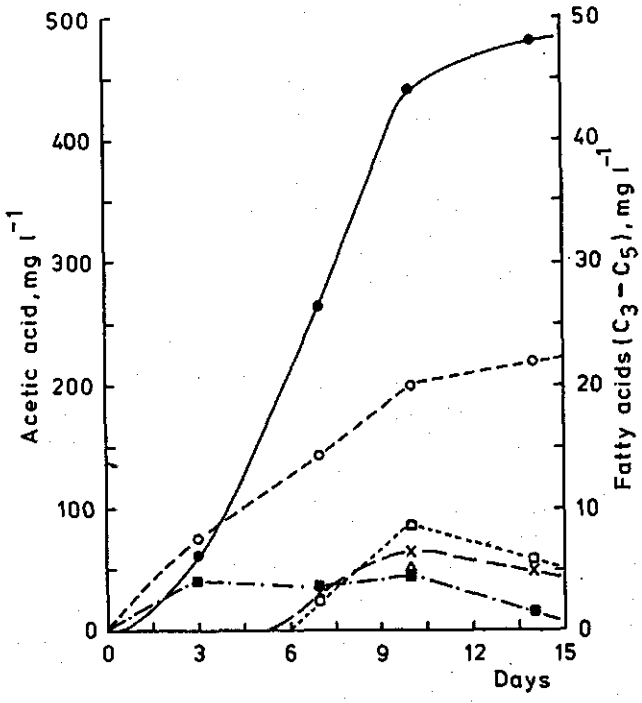

Fig. 7.2. Accumulation of lower fatty acids in the overlying water of sediment incubated anaerobically at 25°C in the absence of nitrate.
●———● Acetic acid; ○– – –○ Propionic acid; X– –X Isobutyric acid; ■—.—■ Butyric acid; □.....□ Isovaleric acid; △ Valeric acid.

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

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

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

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

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,

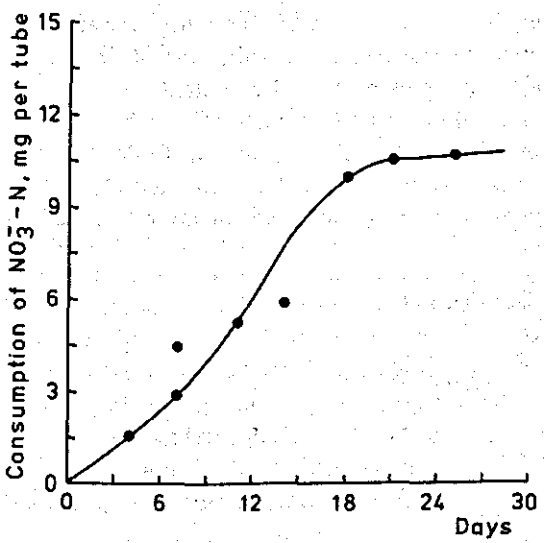

Fig. 7.3. Nitrate nitrogen consumed by the sediment of Experiment 2 during anaerobic incubation at 25°C.

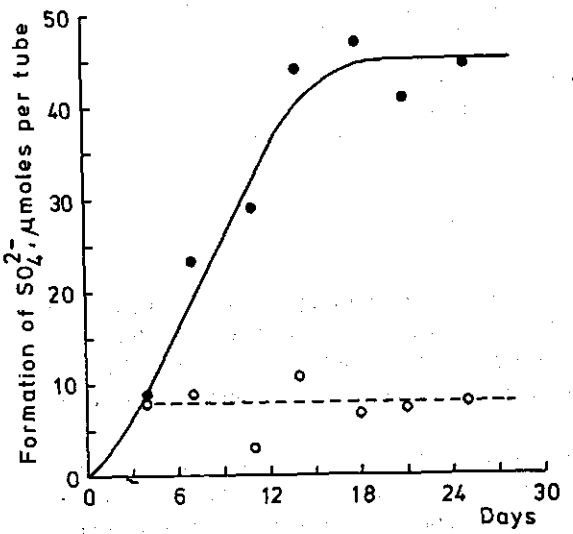

Fig. 7.4. Sulphate in the overlying water of a sediment incubated anaerobically at 25°C in the presence and absence of nitrate. •——• With nitrate; o—o Without nitrate.

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.

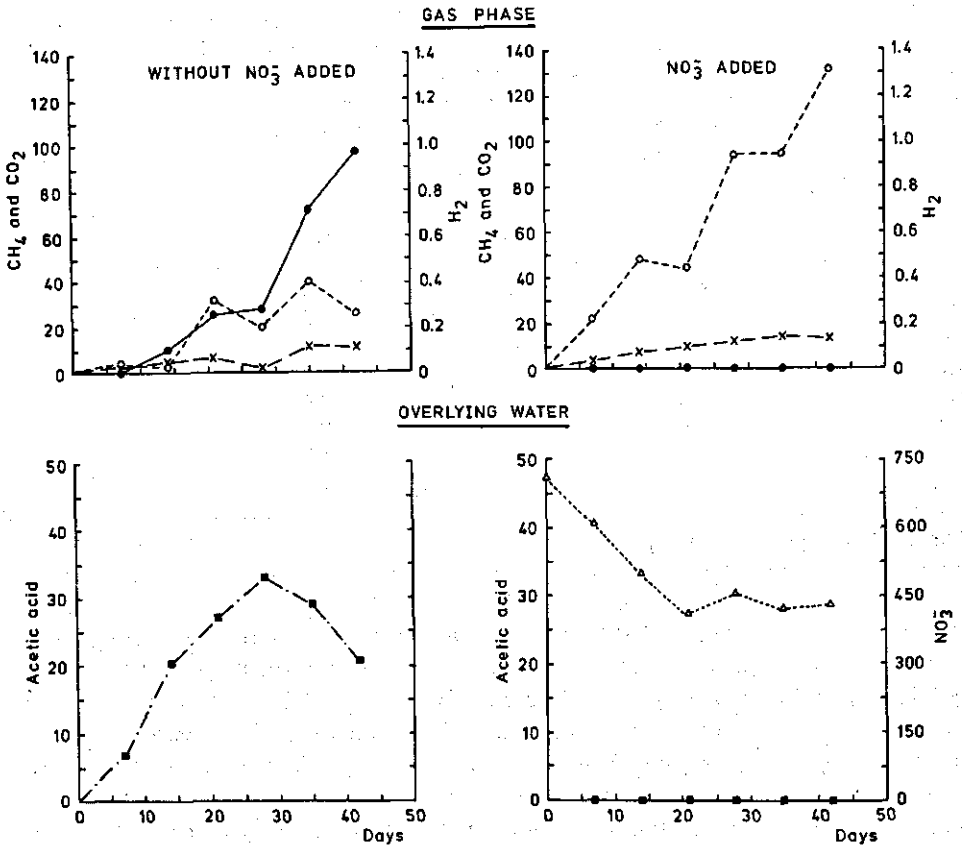

Fig. 7.5. H_2, CH_4 and CO_2 in the gas phase and nitrate and acetic acid in the overlying water (μmoles per tube) of two anaerobic water-sediment systems with and without added nitrate. ●———● CH_4; o - - - - - o H_2; X— —X CO_2; △.....△ Nitrate; ■—.—■ Acetic acid.

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

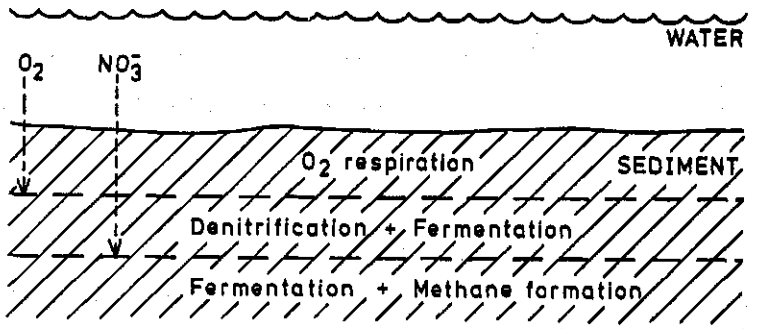

Fig. 7.6. Occurrence of three zones with different microbial activity in a sediment with overlying water containing nitrate as well as oxygen.

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.

Introduction

Purification of domestic and industrial waste water mostly consists of reducing the biochemical oxygen demand. The inorganic nutrients, including nitrogen and phosphorus remain in the effluent mostly in sufficiently large amounts to enable the induction of an enormous growth of algae and aquatic plants after discharging the effluent on surface water (Vollenweider, 1968). The same applies to drain water from agricultural areas, which may contain high concentrations of nitrate (Kolenbrander, 1969; Williams, 1970).

Growth of algae and aquatic plants in surface waters can be limited by minimizing one of their growth factors in the water to be discharged. In the practice of waste water purification this has resulted into the development of methods for removal of nitrogen (Reeves, 1972), phosphorus (Jenkins *et al.*, 1971), or both, from effluents. Introducing these methods into the existing purification plants usually requires a high investment. The indirect pollution of surface waters by discharging nitrogen-containing drain water from agricultural areas has led to the propagation of the opinion that the use of fertilizers should be reduced or even abolished (Commoner, 1971).

In previous laboratory investigations (Chapters 2 and 5) it was shown that there is an interaction between surface water and its sediment, resulting into the removal of nitrate from the surface water by the activity of denitrifying bacteria in the sediment. About 95% of the nitrate nitrogen which is removed from the surface water, disappears from the system as molecular nitrogen into the air, while the remainder is immobilized in the sediment as cell material (Chapter 3).

The ultimate effect of the denitrification process on the total amount of nitrogen in surface waters depends on the rate of denitrification in the sediment and on the ratio of

water volume and wet bottom area. In the Netherlands this ratio in lakes, rivers, canals and ditches is usually small. In this case the removal of nitrate from surface waters by denitrification in sediments can be of great importance and possibly can solve the problems as mentioned in the second paragraph. This was tested in a field experiment in combination with a laboratory experiment.

Materials and methods

Field experiment

Effluent of the purification plant of Goor was used. The plant consists of two primary settling tanks, two aeration tanks for activated sludge and two final settling tanks. It has a design capacity of 120,000 population equivalents. The plant was actually loaded with about 80,000 population equivalents of which about 85% originated from industry and 15% from the population. During the experiment the dissolved oxygen concentration in the activated sludge tanks was automatically controlled and maintained between 1.5 and 2.5 mg l^{-1}, which prevents the occurrence of denitrification. The BOD_5 of the effluent 24-hours settling period. The clear effluent is discharged into a newly dug canal (Fig. 8.1) over a sill (Fig. 8.2). A cross-section of the canal is shown in Fig. 8.3. During the experiment the water depth in the canal was maintained by means of an adjustable weir. The growth of plants in the canal was nil. The upstream flow of the canal consists of drain water from a small agricultural area. The flow rate by which the effluent is discharged, is measured

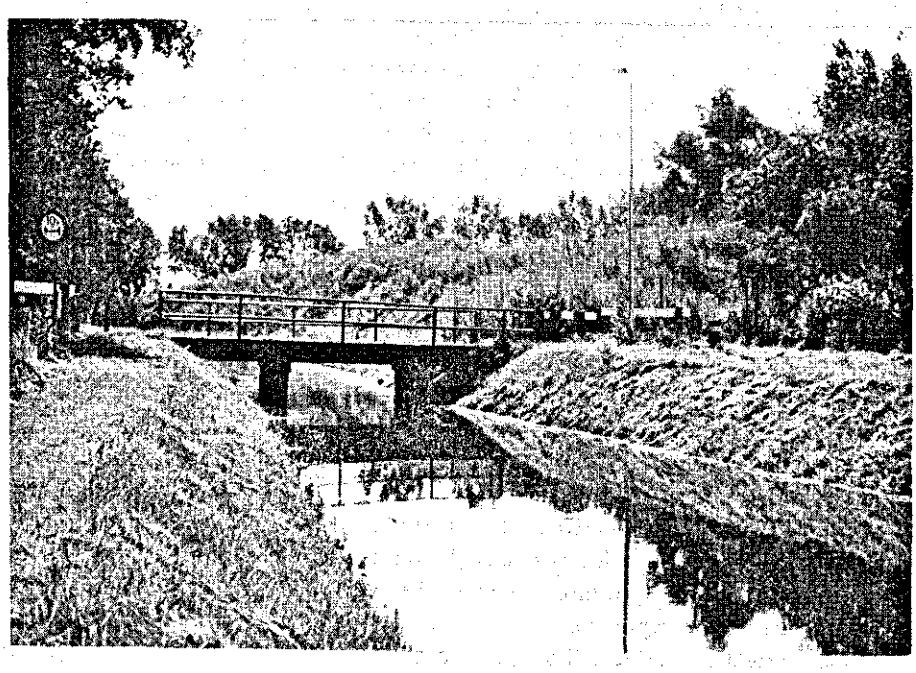

Fig. 8.1. A view of the canal.

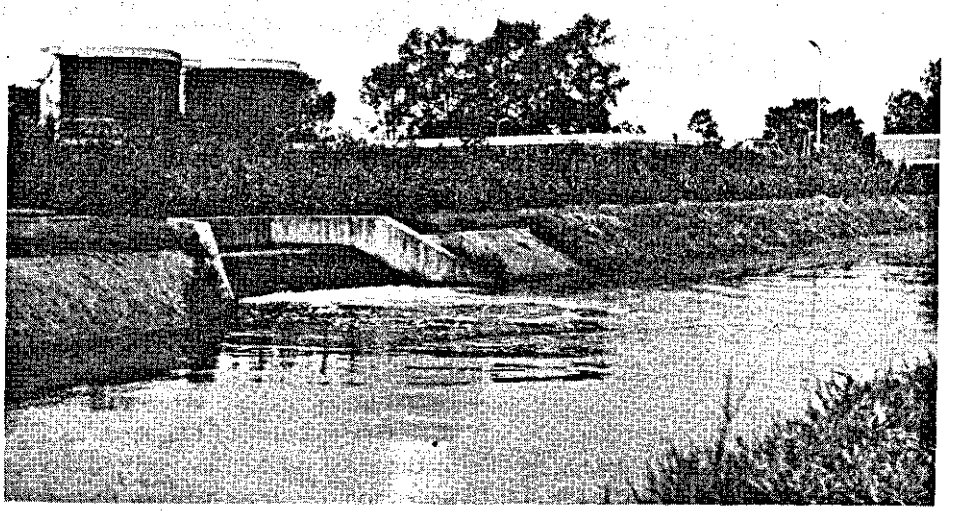

Fig. 8.2. The effluent draining into the canal by a sill.

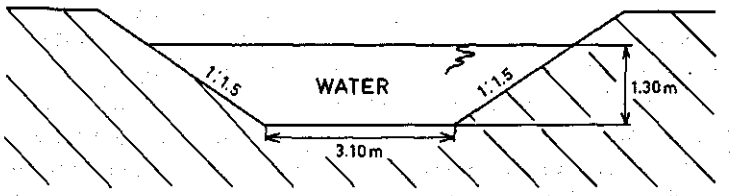

Fig. 8.3. Cross-section of the canal.

with an Altoflux electromagnetic flowmeter (Reinhold, 1974), automatically integrated and continuously recorded.

After two preliminary experiments in which the experimental procedure was improved, the final set-up was as follows. The effluent and the canal water were continuously sampled by three small pumps, each with a flow rate of 2 l h^{-1}. The canal water was sampled at two stations 30 and 830 meters downstream the sill, respectively. The samples were drawn from the middle of the canal at a depth of 0.60 m. The water of each sampling pump was collected in a 50–1 container, directly acidified with sulphuric acid to prevent biological activity and mixed with a magnetic stirrer. A sample was taken from the container for chemical analysis daily at 9.30 a.m. Thereafter the container was emptied. In starting the new sampling period, 50 ml 36 N H_2SO_4 was put into the vessel for acidification of the new sample. A snap sample from the canal water 200 m upstream the sill was also drawn daily at 9.30 a.m. The preliminary experiments showed that one daily sample of the canal water upstream the sill was representative for the daily flow,

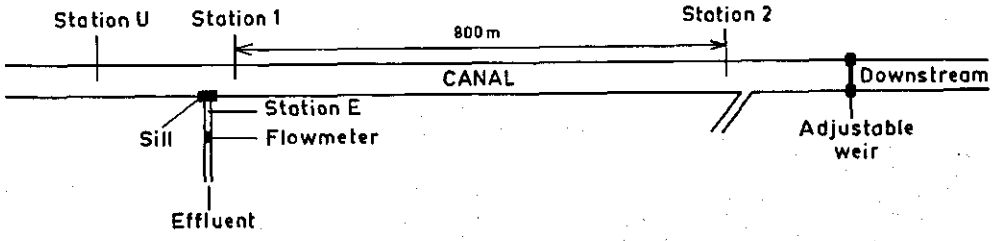

Fig. 8.4. A topographical drawing of the 800-m long reach of the canal with the effluent flowmeter, sill, adjustable weir and sample stations U (upstream), E (effluent), 1 and 2.

because the chloride concentration did not change during a 24-hours period. A map of the canal reach with the four sampling stations is presented in Fig. 8.4. The concentrations of chloride, nitrate, nitrite and ammonium ions were determined in all the collected samples.

The temperature of the water at a depth of 0.60 m and that of the upper layer of sediment were measured daily in the middle of the canal reach. The dissolved oxygen concentration of the water at a depth of 0.60 m at various stations between the sample points 1 and 2 were measured regularly during the experiment.

Laboratory experiment

Half-way the field experiment three undisturbed water-sediment profiles were taken from the middle of the canal bottom between sample stations 1 and 2 by means of a Jenkin mud sampler (Mortimer, 1942). In a preliminary experiment no differences were shown to exist between the water-sediment profiles at various distances downstream sample point 1. The inner diameter of the tubes with the undisturbed water-sediment profiles was 6.9 cm. The thickness of the sediment layer in the tubes was about 15 cm. In the laboratory the volume of the overlying water was brought up to 1000 ml. Five mg N-Serve (2-chloro-6-(trichloromethyl)-pyridine) was added to each tube to inhibit the possible occurrence of nitrite or nitrate production by nitrifying bacteria (Sommer, 1972; Chapter 2). The nitrate-nitrogen concentration of the overlying water was brought up to 6.55 mg l^{-1}. Thereafter the tubes were incubated at 19 ± 0.5°C in a dark room to prevent possible immobilization of nitrate by algae. The pH, nitrate-nitrogen and dissolved oxygen concentrations of the water in each tube were determined daily. Corrections were made for evaporation of water during incubation. The dry weight and the organic matter content of the sediment from the tubes were determined at the end of the experiment.

Chemical analysis

The ammonium-nitrogen concentration of the overlying water was determined with Nessler reagent described in 'Deutsche Einheitsverfahren' (1960). The salicylate method described by Müller & Widemann (1955) was used for the determination of nitrate. Nitrite was analysed by means of the Griess-Romijn-van Eck reagent described in NEN 3252 6.3 (1972). Chloride was determined titrimetrically according to Mohr and

described in Standard Methods (1955). The ammonium and the organic nitrogen contents of the sediment were determined according to Bremner (1965). Dissolved oxygen concentrations were measured with a polarographic oxygen membrane electrode.

Results

Field experiment

The final field experiment was carried out from July 12 until August 1, 1975. During that period no rain has fallen. The temperature of the water at 0.60 m depth and that of the upper layer of the sediment were equal and amounted to 19 ± 1.0°C. The dissolved oxygen concentration of the canal water varied between 3.0 and 6.0 mg l^{-1}. The most important results of the chemical analyses of the collected samples are listed in Table 8.1. The average nitrite-nitrogen concentrations of the samples at stations 1 and 2 were 0.14 and 0.33 mg l^{-1}, respectively. For ammonium nitrogen these values were 0.4 and 0.6 mg l^{-1}. The figures of the nitrate, nitrite and ammonium-nitrogen concentrations of the effluent and those of the upstream canal water are not presented here, because they were only used as a control at station 1. There was a good correlation between these figures and those of station 1.

Table 8.1. The daily chloride concentration (mg l^{-1}) of upstream canal water, the daily flow (m^3) and the daily chloride concentration (mg l^{-1}) of effluent, and the daily nitrate nitrogen and chloride concentrations (mg l^{-1}) of canal water that passed sample stations 1 and 2, respectively.

Date	Upstream canal water	Plant effluent		Station 1		Station 2	
	Cl^- mg l^{-1}	Flow m^3	Cl^- mg l^{-1}	NO_3^-N mg l^{-1}	Cl^- mg l^{-1}	NO_3^-N mg l^{-1}	Cl^- mg l^{-1}
12/7	54	3325	188	8.8	189	3.0	180
13/7	41	2625	177	3.4	174	0.5	189
14/7	69	3150	148	9.9	162	0.2	159
15/7	81	2805	141	3.0	141	1.8	177
16/7	73	3640	128	0.6	131	4.5	143
17/7	63	4620	112	0.7	117	2.0	131
18/7	74	3660	97	0.3	101	0.3	110
19/7	51	4030	82	0.7	91	0.2	99
20/7	53	2435	79	0.7	82	0.0	87
21/7	48	2525	77	2.0	78	1.2	80
22/7	46	2525	80	4.5	78	0.7	81
23/7	42	2740	80	1.6	80	1.8	80
24/7	43	2625	88	0.7	80	1.9	82
25/7	44	2860	84	3.0	86	0.4	87
26/7	45	2215	87	3.9	88	1.8	86
27/7	48	2020	90	3.6	86	0.2	88
28/7	61	4220	95	7.3	96	0.7	90
29/7	60	5105	123	0.5	118	3.0	93
30/7	66	4510	163	6.2	158	2.3	120
31/7	78	4770	205	0.4	202	0.2	158

The sum of the effluent flow (Q_E) and the upstream canal water flow (Q_U) results in the flow of the canal water passing daily at sample station 1 (Q_1) (equation 1).

$$Q_E + Q_U = Q_1 \tag{1}$$

The chloride concentrations of the four daily samples were determined to compile the balance of the daily water flow. It was assumed that chloride is biologically inert in this system, so that the mass flow of chloride at sample station 1 should be the sum of the mass flow of the effluent and that of the upstream canal water (equation 2).

$$Q_E \cdot [Cl^-]_E + Q_U \cdot [Cl^-]_U = Q_1 \cdot [Cl^-]_1 \tag{2}$$

Combination of these two equations results in equation 3 from which Q_1 can be computed with the aid of the data in Table 8.1. The results of these calculations are listed in Table 8.2.

$$Q_1 = \frac{Q_E \cdot ([Cl^-]_E - [Cl^-]_U)}{([Cl^-]_1 - [Cl^-]_U)} \tag{3}$$

The water volume of the 800-m long reach between sample stations 1 and 2 was calculated from the cross-section of the canal (Fig. 8.2) being 5250 m^3. The average

Table 8.2. Q_1 (m^3), the retention time of the canal water in the 800-m long reach, and the amounts of nitrate nitrogen and chloride (kg) that passed sample stations 1 and 2 during 24-hours periods.

Date	Q_1 m^3	Retention time days	Station 1		Station 2	
			NO_3^--N kg	Cl^- kg	NO_3^--N kg	Cl^- kg
12/7	3300	1.6	29.0	624		
13/7	2685	2.0	9.1	467	1.3	507
14/7	2675	2.0	26.5	433	0.5	425
15/7	2805	1.9	8.4	396	5.0	496
16/7	3450	1.5	2.1	452	15.5	493
17/7	4190	1.3	2.9	490	8.4	549
18/7	3115	1.7	0.9	315	0.9	343
19/7	3125	1.7	2.2	284	0.6	309
20/7	2185	2.4	1.5	179	0.0	190
21/7	2440	2.2	4.9	190	2.9	195
22/7	2685	2.0	12.1	209	1.9	217
23/7	2740	1.9	4.4	219	4.9	219
24/7	3195	1.6	2.2	256	6.1	262
25/7	2725	1.9	8.2	234	1.1	237
26/7	2165	2.4	8.4	191	3.9	186
27/7	2235	2.3	8.0	192	0.4	197
28/7	4100	1.3	29.9	394	2.9	369
29/7	5545	0.9	2.8	654	16.6	516
30/7	4755	1.1	29.5	751	10.9	571
31/7	4885	1.1			1.0	772

retention time of the canal water (days) between sample stations 1 and 2 was calculated from the water volume Q_1 (Table 8.2).

In the first instance it was assumed that the flow rate did not change between sample stations 1 and 2. Now it was possible to calculate the mass flow of nitrate nitrogen and Cl^- (kg) that passed both stations during a 24-hours period (Table 8.2). The average retention time at the first day of the experiment (12/7) was 1.6 days, which means that the canal water passing sample station 1 did not arrive at station 2 at the same day. Therefore, the amount of nitrate nitrogen and that of chloride passing sample station 2 at 12/7 were not inserted in the balance. For the same reason the data of nitrate and chloride passing sample station 1 on the last day of the experiment (31/7) were omitted.

The total amounts of nitrate nitrogen and chloride which passed sample station 1 during the 19-days period were 193.0 and 6930 kg, respectively. For station 2 these figures amounted to 84.8 and 7053 kg. If the canal water was not diluted between the sample stations 1 and 2, then the amount of chloride passing both stations during the experimental period should be equal. This assumption was made earlier and proved to be a rather good one (Table 8.2). Thus during this period 108.2 kg nitrate nitrogen, 56% of the load, disappeared from the canal water which had an average retention time of 1.7 days within the 800-m long reach of the canal.

The sediment surface of the 800-m long reach was calculated from the cross-section of the canal (Fig. 8.3) and amounted to 6240 m^2. From this figure, the amount of nitrate nitrogen disappearing during the experimental period, and the average retention time of the canal water within the 800-m long reach, the average rate of nitrate-nitrogen removal during the 19-days period was calculated and was found to amount to 537 mg NO_3^--N m^{-2} day^{-1}.

Laboratory scale experiment

The measurements in this experiment were carried out during 4 days. The dissolved oxygen concentration of the overlying water never dropped below 3.5 mg l^{-1}. The pH of the overlying water varied between 7.4 and 8.1. The average results of the daily nitrate-nitrogen determinations are given in Fig. 8.5.

Earlier investigations (Chapter 2) showed that the denitrification rates in the sediment were depending on the nitrate-nitrogen concentration in the overlying water. The denitrification rates in sediment of the canal at various nitrate-nitrogen concentrations in the overlying water were computed from Fig. 8.5. These values were plotted versus the corresponding nitrate-nitrogen concentrations in the overlying water (Fig. 8.6). The dry weight and the organic matter content, the latter determined as volatile solids, were 145 and 25 mg per g of wet sediment, respectively, at the end of the experiment.

Discussion

The chloride balance of the experiment did not fit completely (Table 8.2). Sometimes the calculated daily flow of canal water at station 1 was smaller than that of the effluent. This certainly was the result of errors in the sampling procedure. These errors were caused by the daily variation of the discharge of the effluent. The low upstream canal water flow was a result of a long dry period during which no drain water from the agricultural area

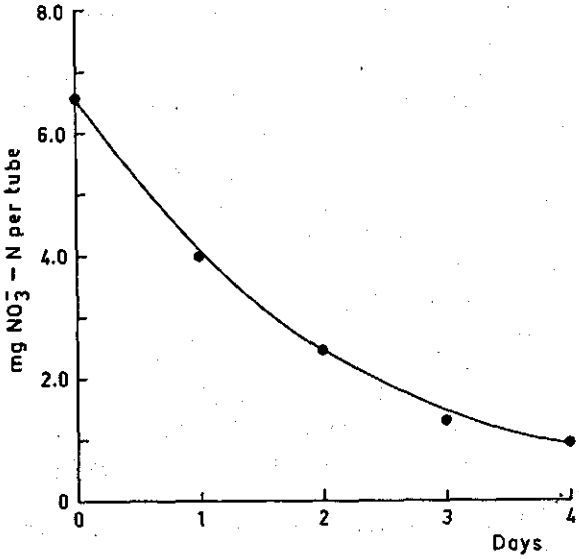

Fig. 8.5. The disappearance of nitrate nitrogen from overlying water of undisturbed water-sediment profiles taken from the canal.

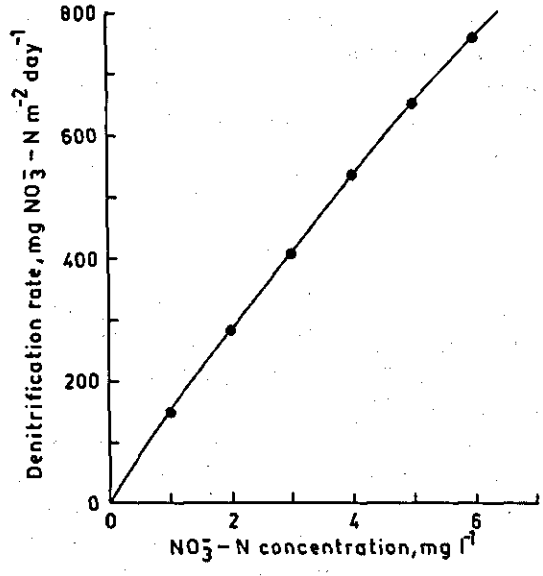

Fig. 8.6. The relation between the denitrification rate in the sediment and the nitrate-nitrogen concentration of the overlying water in undisturbed water-sediment profiles taken from the canal.

was discharged. The mass flow of chloride at station 2 was slightly higher than that at station 1, which was probably caused by evaporation of canal water during its stay (on an average 1.7 days) in the 800-m long reach.

In spite of these imperfections in the water balance it will be clear that the removal of nitrate nitrogen from the canal water during its flow through the 800-m long reach of the canal was significant (Table 8.2). Almost 56% of the amount of nitrate nitrogen that passed sample station 1 disappeared. In preliminary experiments the same results were obtained.

The nitrate-nitrogen concentration of the canal water that passed sample station 1 varied between 0.3 and 9.9 mg l^{-1} (Table 8.1). From the result of the laboratory

experiment it was calculated that the denitrification rates corresponding with the nitrate-nitrogen concentrations varied between 65 and 1100 mg NO_3^--N m^{-2} day^{-1}. In natural waters these values will be higher because the supply rate of nitrate to the sediment is higher in nature (Chapter 2). In sediment of an English river Edwards & Rolley (1965) found denitrification rates varying between 100 and 1500 mg NO_3^--N m^{-2} day^{-1}. It is assumed that the nitrate nitrogen disappearing from the canal water during its flow through the 800-m long reach of the canal was mainly a result of the denitrification process. Only a small proportion could be resulted from immobilization of nitrate by phytoplankton. Denitrification occurred only in the sediment, because this process was inhibited in the canal water by the presence of dissolved oxygen.

From these experiments it is concluded that the natural removal of nitrate from surface waters by denitrification in sediments is of great practical importance. Also Kaushik *et al.* (in press) showed in a 2-km long, springfed small Ontario stream, that denitrification in the stream sediment may act as an important permanent nitrogen sink.

Increased knowledge of this phenomenon may lead to an effective and cheap solution of many nitrogen-induced eutrophication problems in practice. These situations will result from non-point discharges of drain water from agricultural areas as well as from effluent discharges of waste water purification plants.

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9A Respirometer experiments. 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.*

Introduction

The respiration of microorganisms is usually measured by the manometric technique of Warburg. Since the development of the oxygen-membrane electrode many investigators are using this electrode for measuring the oxygen consumption in respiration experiments.

Many bacteria are capable of utilizing nitrate as terminal electron acceptor under anaerobic conditions. Nitrate reduction during this process can proceed to different levels, *viz.* nitrite, nitric oxide, nitrous oxide, molecular nitrogen or ammonium. The production of these compounds depends on the type of bacterium and on the growth conditions of the organisms. The formation of gaseous products from nitrate is called denitrification.

The utilization of nitrate as terminal electron acceptor can be measured by determining the decrease of the nitrate concentration or the increase of the concentration of the end products resulting from the reduction reactions. The chemical analysis of these compounds is rather laborious and usually requires large samples. Therefore, the application of a nitrate-specific ion electrode for continuously measuring the nitrate concentration without altering the culture volume, would be profitable.

This paper describes the results of an investigation on the combined use of an oxygen-membrane electrode and a nitrate-specific ion electrode in one test vessel. The developed respirometer has been tested with *Ps. aeruginosa* strain P8.

Materials and methods

Description of the respirometer

The respirometer, which permits simultaneous measurements of the oxygen and nitrate concentrations, is shown in Figs. 9A.1 and 9A.2. The apparatus is made from polymethyl methacrylate. The volume of the sample chamber is 100 ml. The temperature in the sample chamber can be controlled by the presence of a water-jacket. The oxygen and reference electrodes are sealed in the roof of the sample chamber with two rubber 'O' rings to prevent oxygen diffusion from the atmosphere into the sample chamber. The nitrate electrode is fixed into a stopper which contains also two 'O' rings. The outside of the stopper is slant and is provided with an access slot for removal of overflowing liquid

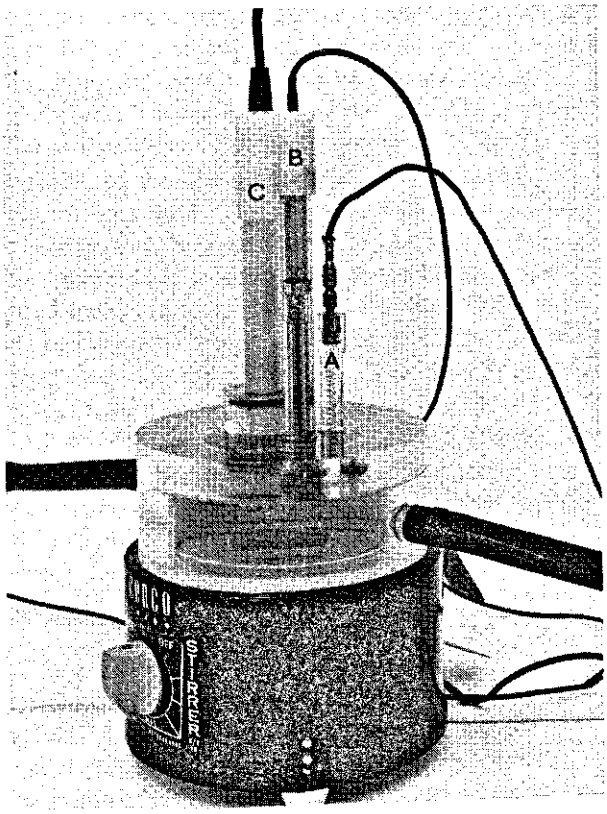

Fig. 9A.1. Respirometer.
(A) Oxygen-membrane electrode
(B) Reference electrode
(C) Nitrate-specific ion electrode

and gas bubbles from the sample chamber. The roof of the sample chamber is spherical to facilitate the removal of gas bubbles. The removal of air bubbles is very important because air contains much more oxygen per unit of volume than an aqueous solution saturated with oxygen. The length of the access slot should be rather extensive because the small amount of unstirred solution in the slot serves as a barrier preventing oxygen diffusion into the stirred solution. Because measurements take place during short times, the error caused by diffusion of oxygen is negligible.

The access slot can also be used for adding chemicals to the sample. No measurable dilution or temperature effect has been observed by adding 10–100 μl of a solution to the 100-ml sample.

Determination of dissolved oxygen

The oxygen concentration is measured with a Beckman Oxygen Macro Electrode connected with a Beckman model 160 Physiological Gas Analyser. The latter is coupled with a recorder. The used oxygen electrode is a polarographic sensor, which permits measurements of very low oxygen tensions in liquids if the electrode is modified as described by Koch and Kruuv (1972). The oxygen electrode is sensitive to temperature changes. Therefore, it is important to keep the temperature in the sample chamber

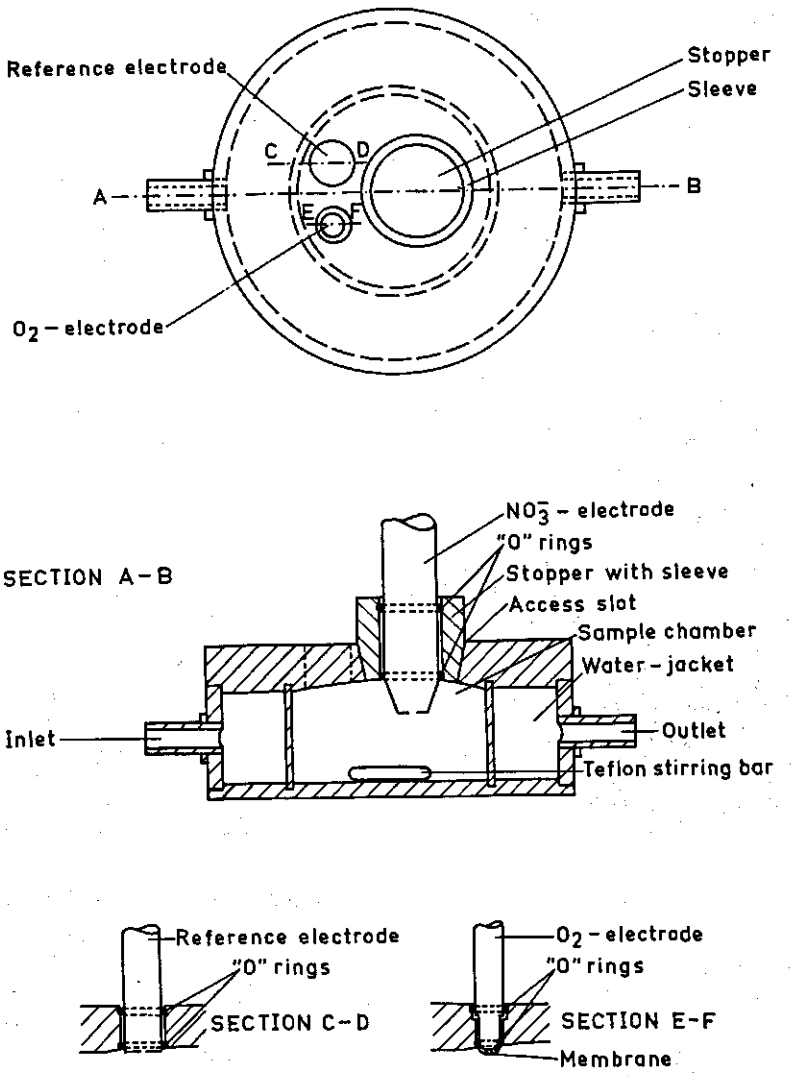

Fig. 9A.2. Scheme of the respirometer.

constant. The oxygen-membrane electrode must be calibrated by establishing two known reference points on the recorder.

Determination of nitrate ions

The nitrate electrode, Orion model 92–07, is coupled with an Orion Ionanalyser model 407, which is connected with a recorder. This electrode is used in connection with a Double Junction Reference Electrode, model 90–02. The outer chamber of this reference electrode is filled with 0.25 M Na_2SO_4.

Both electrodes are described in the Instruction Manual (1971). The nitrate electrode

detects nitrate ions by developing a potential across a thin layer of a water-immiscible liquid ion exchanger. The ion exchanger, which is most selective for nitrate ions, responds also to other anions. In these experiments nitrite is an important interfering anion, because part or all of the nitrate may accumulate as nitrite during denitrification. Therefore, it is necessary to determine nitrite chemically. This is done by the method of Griess-Romijn-van Eck described in NEN 1056 IV.2 (1966). On the other hand it is also possible to use the nitrate electrode for measurements with nitrite as the only terminal electron acceptor. The calibration of the nitrate electrode was carried out before every measurement. It appeared to be inaccurate to make an absolute calibration curve, because the electrode is sensitive for changes in temperature, interfering ion concentrations, and total ionic strength. Therefore, the nitrate electrode is calibrated in the cell suspension to be investigated. It appeared to be necessary that this suspension contains more than 3 mg O_2 l^{-1} during the calibration. The latter is performed by step-wise additions of known amounts of nitrate. The Ionanalyser is switched on the exponential scale and connected with a recorder with a 0–5 or 0–10 mV (logarithmic) scale. In experiments with nitrite as terminal electron acceptor, the calibration curve is set up with nitrite. By using equimolar amounts of nitrate and nitrite, the nitrate electrode is more sensitive to nitrate than to nitrite.

Cultivation of Ps. aeruginosa

Washed cells of *Ps. aeruginosa*, strain P8, were used to investigate the suitability of the developed respirometer. This strain has been isolated and described by Woldendorp (1963). It accumulates only gas and no nitrite or ammonium during nitrate respiration under the conditions tested. The organism was cultivated in 300-ml Erlenmeyer flasks containing 200 ml of the following medium (Woldendorp, 1963): tryptose, 10 g; beef extract, 3 g; yeast extract, 2 g; glucose, 10 g; K_2HPO_4, 5 g; KNO_3, 10 g; tap water, 1 litre; pH 7.0. The flasks were inoculated with a culture maintained on pepton-glycerol-agar slopes and incubated at 30°C without shaking. The cells were harvested during the exponential growth phase and washed twice with 0.005 M phosphate buffer, pH 7.0. Subsequently, the cells were resuspended in a 0.005 M phosphate buffer, pH 7.0, containing 10 g glycerol and 50 mg NH_4Cl l^{-1} and trace elements. About 100 ml of this suspension containing approximately 0.9 mg cells (dry weight) per ml was adjusted to 30°C and then transferred to the sample chamber.

Results and discussion

Accumulation of nitrite by *Ps. aeruginosa* did not occur during denitrification with nitrate as terminal electron acceptor.

The oxygen concentration of the bacterial suspension decreased linearly with time and became nil 14 min after the start of the experiments (Fig. 9A.3). The rate of oxygen consumption was 1.13 μmol 100 ml^{-1} min^{-1}.

The nitrate consumption started immediately after the oxygen was exhausted (Fig. 9A.3). The nitrate concentration also decreased linearly with time. The rate of nitrate consumption was 0.46 μmol per 100 ml min^{-1}. By using nitrite as terminal electron acceptor, instead of nitrate, nitrite consumption also started immediately after the oxygen was used

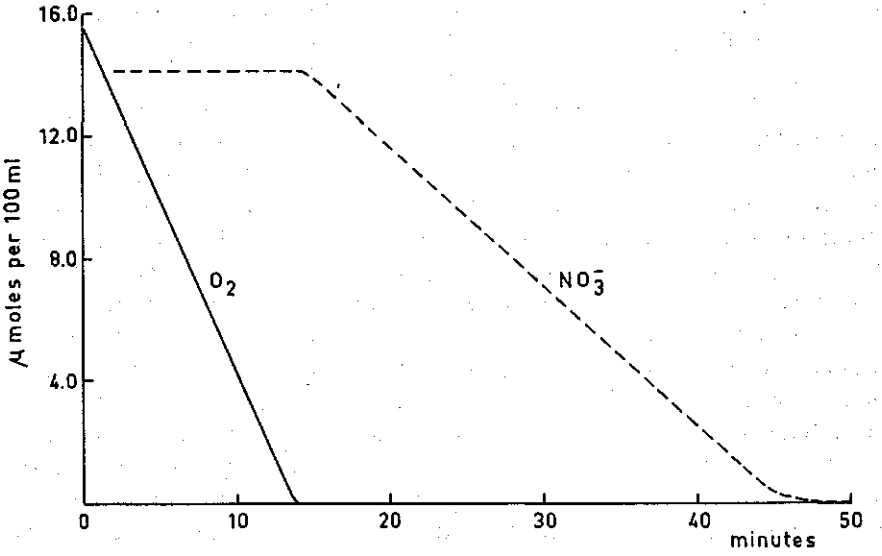

Fig. 9A.3. Respiration of washed cells of *Ps. aeruginosa* on glycerol with oxygen or nitrate as terminal electron acceptor.

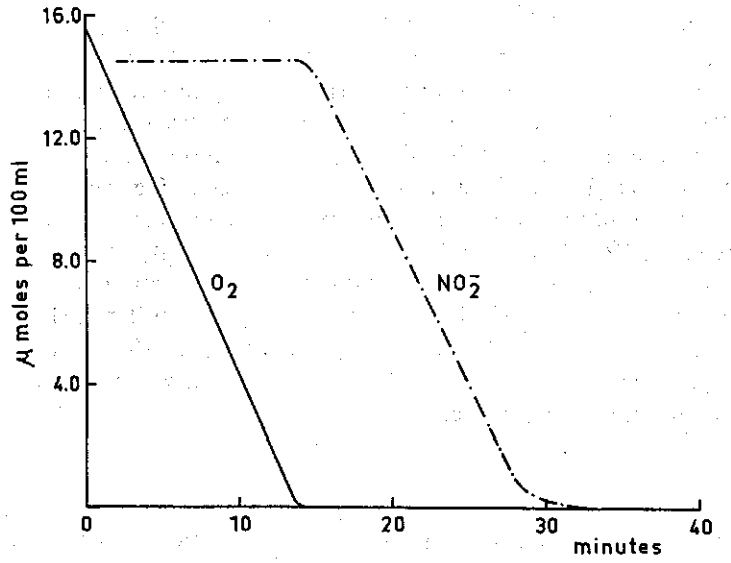

Fig. 9A.4. Respiration of washed cells of *Ps. aeruginosa* on glycerol with oxygen or nitrite as terminal electron acceptor.

up and the rate of nitrite consumption amounted to 1.04 μmol per 100 ml of bacterial suspension per minute (Fig. 9A.4).

In the respirometer experiments with nitrite, the specific nitrate electrode potential did not reach the base line, although chemical analysis showed that nitrite was absent. In

the Instruction Manual (1971) it is stated, that if the electrode is placed in a nitrate-free solution containing any other ion to which the electrode responds, the interfering ion gradually replaces the nitrate ion in the ion exchanger. This observation suggests that nitrite partly replaces nitrate and that this process caused the above-mentioned deviation.

During the work with this respirometer it appeared that the wall of the sample chamber had a reduced conduction of heat. This disadvantage may partly be eliminated by making the wall from glass or steel.

From the above-mentioned experiments it may be concluded that the respirometer described in this paper has the same possibilities as those commonly used. However, it has the advantage of measuring nitrate and oxygen simultaneously. It only requires a relatively large sample.

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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

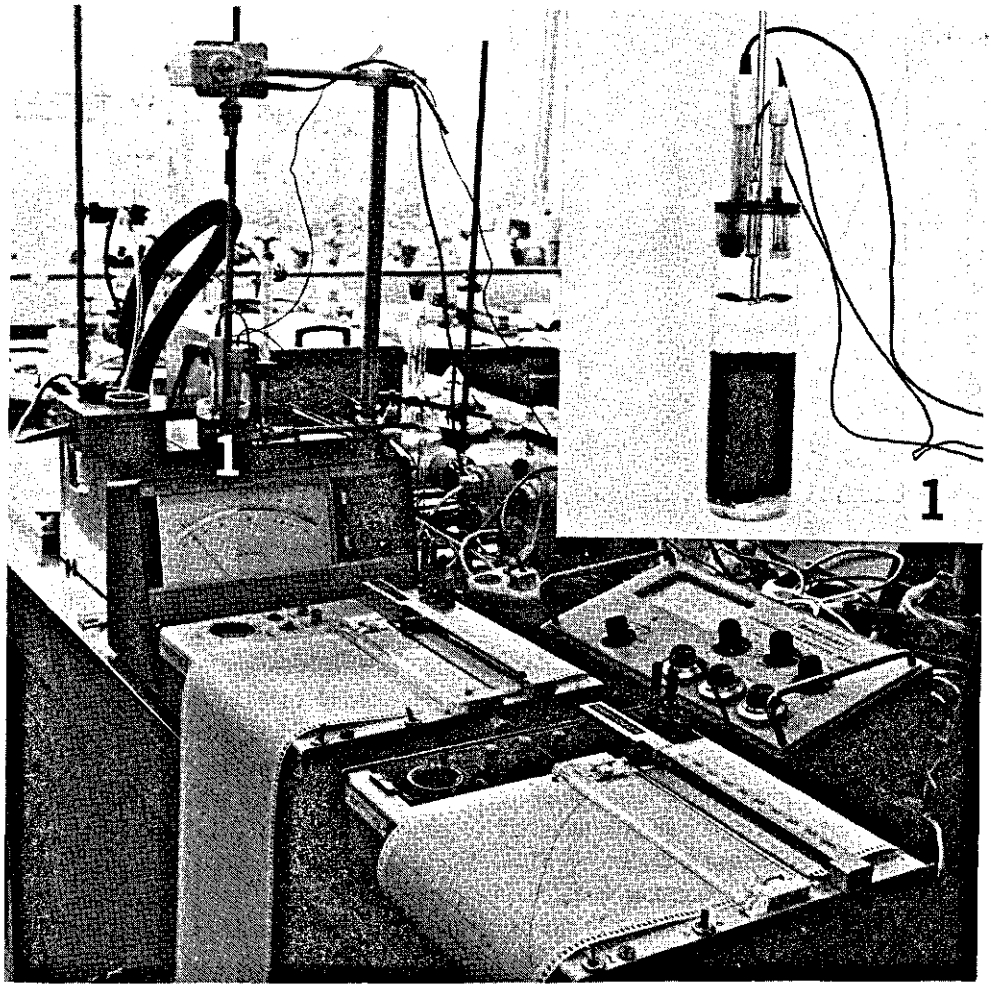

Fig. 9B.1. The measuring system for oxygen and nitrate uptake by undisturbed sediment cores.

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

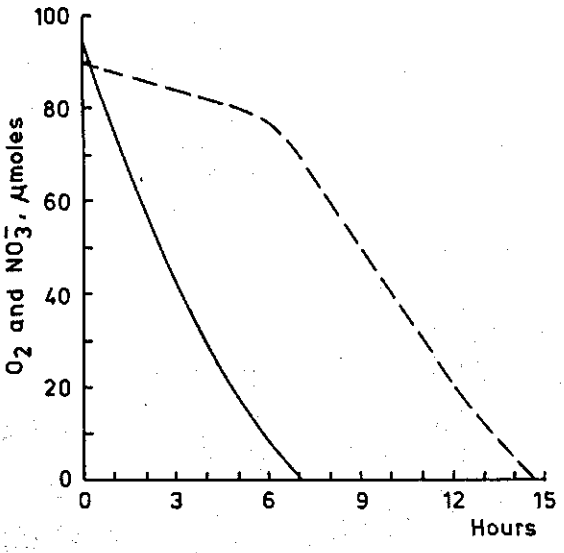

Fig. 9B.2. Amount of O_2 and NO_3^- (µmoles) in the overlying water of an undisturbed water-sediment core (area of sediment surface: 30 cm^2). ——O_2; – – – NO_3^-.

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)

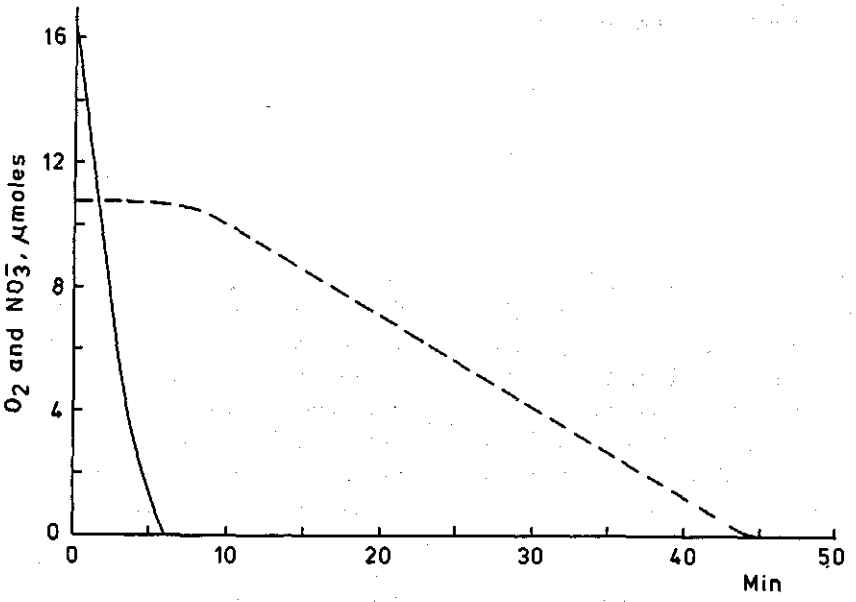

Fig. 9B.3. Amounts of O_2 and NO_3^- (*µmoles*) in a completely mixed suspension derived from 50 g wet sediment.
——O_2; – – – NO_3^-.

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.

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 de 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.