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ECOLOGICAL ASPECTS OF THE BIOLOGICAL PHOSPHATE REMOVAL FROM WASTE WATERS

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Proefschrift

ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
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van de Landbouwuniversiteit te Wageningen.

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Stellingen

1. De aanduidingen "Biologische defosfatering in de sliblijn" en "Biologische defosfatering in de waterlijn" dienen vervangen te worden door "Biologische fosfaatverwijdering in de hoofdstroom" en door "Biologische fosfaatverwijdering in de zijstroom" in verband met de duidelijkheid over de procesvoering.

2. Gezien het feit dat in Nederland reeds jarenlang Engelse afkortingen als DNA (Desoxyribonucleïnezuur) in plaats van DNZ ingeburgerd zijn, lijkt het logisch om universeel BOD (Biologisch zuurstofverbruik) en COD (Chemisch zuurstofverbruik) in plaats van BZV en CZV te gaan gebruiken.

3. De verplichting om stellingen toe te voegen bij proefschriften op de Landbouwuniversiteit wordt vermoedelijk veroorzaakt doordat deze hogeschool zich pas enige jaren universiteit mag noemen.

4. De toepassing van het RIM-NUT proces om fosfaten en ammonia uit afvalwater te verwijderen zal leiden tot een sterke verzouting van het milieu.
Liberti et al. 1988. Role of nitrates and sulfates during wastewater treatment by the RIM-NUT process. Trib. Cebedeau 41:27-35

5. Hoe langer snij-bloemen blijven staan, hoe eerder zij als chemisch afval zijn te beschouwen.

6. De controle op loslopende honden zou zodanig verscherpt moeten worden dat iedereen veilig in het vrije veld kan recreëren.

7. Met de aanwezigheid van protozoa onder denitrificerende omstandigheden is nog niet aangetoond dat deze organismen nitraat als electronenacceptor kunnen gebruiken.
A. Grabińska-Loniewska. 1991. Denitrification unit biocenosis. Water Res. 25:1565-1573.

8. Bij het verbouwen van een huurhuis door een huurder dient de verhuurder niet de problemen van herstel naar de oude toestand door te schuiven naar de volgende huurder.

9. De verschuiving van de fermentatiebalans in aanwezigheid van nitraat, waardoor de acetaat-vorming door o.a. *Escherichia coli* groter wordt, doet veronderstellen dat nitraat een positief effect kan hebben op de fosfaatafgifte door biologisch defosfaterend slib.

R. Rehr and J.H. Klemme. 1989. Competition for nitrate between denitrifying Pseudomonas stutzeri and nitrate ammonifying enterobacteria. FEMS Microbiol. Ecol. 62:51-58.

10. De conclusie "In spite of all we learn and understand, some sludges will still bulk" geeft de moeilijkheidsgraad van het onderzoek naar het actief-slibproces aan.

O.E. Albertson. 1991. Bulking sludge control-progress, practise and problems. Water Sci. Tech. 21:835-846

11. In tegenstelling tot hetgeen Kayser toont, kan de stikstofverwijdering in een waterzuiveringsinstallatie nooit 100% worden bij oneindige recirculatie van slib over anaërobe en aërobe zones

R. Kayser 1983. Ein Ansatz zur Bemessung einstufiger Belebungsanlagen für Nitrifikation-Denitrifikation. gwf-Wasser/Abwasser 124:419-427.

12. Het tonen van een onbegrijpelijke formule en ervoor verwijzen naar een persoonlijke mededeling in de referentie-lijst is niet bevorderlijk voor de geloofwaardigheid van het stikstofverwijderingsmodel van Kayser

R. Kayser. 1983 Ein Ansatz zur Bemessung einstufiger Belebungsanlagen für Nitrifikation-Denitrification. gwf-Wasser/Abwasser 124:419-427.

13. Het eiwit-gehalte per g drogestof (0,13 g/g) van de *Acinetobacter*-stammen bestudeerd door Vasiliadis et al. is ongezond laag.

G. Vasiliadis et al. 1990. Polyphosphate production by strains of Acinetobacter. FEMS Microbiol. lett. 70:37-40.

14. Gezien het feit dat de Kluyver-prijs alleen gewonnen kan worden door jonge promovendi, dient er een prijs te komen voor de oudere jongere promovendi.

Stellingen behorende bij het proefschrift 'Ecological aspects of the biological phosphate removal from waste waters'. K.J. Appeldoorn, Wageningen, 13 april 1993.

CONTENTS

Chapter 1

General introduction

GENERAL INTRODUCTION

1. THE ROLE OF PHOSPHATE IN THE EUTROPHICATION

Eutrophication refers to a situation where an increase in nutrient levels has occurred through anthropogenic activities which has resulted in "nuisance" algal growth (Bell 1992). "Nuisance" categories include (1) perceptible water quality deterioration, including trophic changes; (2) chronic or intermittent health hazards, including toxicity; and (3) loss of aesthetic and hence recreational values. In most fresh water environments phosphorus is the limiting nutrient controlling eutrophication (Boers and Zevenboom 1992). Nitrogen is typically the limiting nutrient in estuarine and marine waters (Randall 1992). In the North Sea algae blooms are especially regulated by the availability of nitrogen (Boers and Zevenboom 1992). To prevent eutrophication by phosphorus and nitrogen measures have to be taken to reduce the input of both nitrogen and phosphorus into the environment.

2. SOURCES OF PHOSPHORUS EMISSIONS

There are three main sources of phosphorus emission to the environment: Agriculture, industry and human excretions. In Western Europe, domestic waste waters contained 12 to 20 mg P/l in 1987. In North-America and Japan sewages were more diluted, and phosphorus concentrations ranged between 2 and 10 mg/l (Meganck 1987). 2 g phosphorus per person per day originating from human excretions has been estimated to be released into the environment (Dijk 1989). In 1984, the use of detergents contributed an additional 2 g phosphorus per person per day in The Netherlands.

To reduce the phosphorus input from domestic origin, the phosphorus compounds have been replaced in detergents by substitutes. However, the phosphorus load originating from

human metabolism is often high enough to continue eutrophication even with detergent being completely banned (Berthouex et al. 1985, Meganck 1987). In The Netherlands it is planned to reduce the effluent phosphorus concentration of wastewater treatment plants in 1995 by about 50% of the concentration in 1988 (De Jong and Van de Velde 1988). After 1995 the discharge demands will become even more stringent depending on the location and magnitude of a wastewater treatment plant. Average phosphorus emissions of treatment plants have to become 0.5 mg P/l, when draining into stagnate surface waters (Van Starkenburg 1988). Standards for draining into other surface waters become 2 mg P/l for plants treating waste water of 100,000 p.e. (population equivalents) and 1 mg P/l for plants treating waste water of more than 100,000 p.e. (Anonymous 1991). In 1988 about 5% of the Dutch treatment plants were equipped for the removal of phosphate (Bakker 1988). Many treatment plants have still to be adapted to meet the new phosphorus effluent standards by 1995.

3. THE ACTIVATED SLUDGE PROCESS

The activated sludge process was developed in England (Ardern and Locket 1914). In this process organic waste is introduced into a tank, where a bacterial culture is maintained in suspension by mechanical mixing, or mixing with air or pure oxygen by aerators. This keeps the environment in the tank oxic. The bacteria in the aerated tanks form sludge flocks. Other microorganisms also present, such as protozoa, are believed to have a function in maintaining the flocculated organization by grazing particularly on free bacteria (Metcalf and Eddy 1991). The organic waste is used by heterotrophic bacteria for growth and dissimilatory processes. After a specific period of residence in the aeration tank, the mixture of sludge and water flows into a clarifier, in which the sludge settles and purified effluent leaves the process. Most of the settled sludge is recirculated to the influent part of the aeration tank. This sludge is called return sludge. A

small portion of the sludge is wasted as surplus sludge, which corresponds to the bacterial growth resulting from the influent substrates. In this way the desired concentration of organisms is maintained in the aeration tank.

In many cases an activated sludge plant is equipped with a so-called primary clarifier. Here, the readily settleable organic plus inorganic solids and floating material are removed. Primary clarifiers allow smaller aeration tanks for waste water treatment.

Nitrogen compounds can be eliminated in the activated sludge process by means of nitrification and denitrification. During nitrification, bacteria convert ammonium aerobically into nitrate. Nitrate can be reduced to gaseous nitrogen compounds by denitrifying bacteria in the absence of oxygen.

4. CHEMICAL PHOSPHORUS REMOVAL FROM WASTE WATERS

In The Netherlands conventional treatment plants extract about 40% of the 15 mg P/l from the waste waters (Dijk 1989). This removal is largely due to the growth of the biomass in the sludge. Additional phosphorus can be precipitated and consequently eliminated from waste water with calcium, iron or aluminium salts (Yeoman et al. 1988; Meganck and Faup 1988). Chemical precipitation has several disadvantages:

- The costs increases due to the use of chemicals;
- Precipitated metal phosphates increase surplus sludge production. In addition, surplus sludge is polluted with added metals and heavy metals, which are present as contaminants in the salts;
- The addition of metal salt increases the salinity of the effluent of wastewater treatment plant;
- Without additional measures to reduce phosphorus emissions, it is difficult to attain effluent concentrations < 1 mg P/l with chemical precipitation, which might become a problem when more stringent effluent demands must be met (Tessel 1988; De Jong and Van Starkenburg 1989).

5. BIOLOGICAL PHOSPHORUS REMOVAL FROM WASTE WATERS

Besides elimination of phosphate by precipitation, phosphate can be biologically removed in the activated sludge process. Phosphate can be simply removed due to microbial growth and as such be linked stoichiometrically to the increase of biomass during the treatment process. Phosphate can also be taken up in excess of what is strictly needed for growth. The excess phosphate is stored intracellularly as polyphosphate. Bacteria forming polyphosphate are enriched if the activated sludge is exposed alternatingly to anoxic and oxic conditions. Less than 1 mg P/l in the waste water effluent can be attained by this so-called "enhanced biological phosphate removal" (Yeoman et al. 1988).

Many process configurations have been designed for enhanced biological phosphate removal. Some configurations are described below in some detail.

The conventional full scale plants exhibiting enhanced phosphate removal are long plug flow reactors, operating under high organic loading rates (0.2-0.7 kg BOD/kg sludge dry weight.d) or equivalently with very low sludge ages (1.5-6 days; Marais et al. 1983). Return sludge is mixed with waste water at the influent side of the reactor. The first part of the reactor is not aerated allowing anaerobiosis to occur. Only the second part is aerated. During the initial anoxic phase, excess phosphate is released by the sludge and subsequently in the oxic phase taken up again together with influent phosphate (Fig. 1). Because of the formation of polyphosphate, aerobic phosphate uptake is much higher than would expected solely by sludge growth. By withdrawal of surplus sludge with the higher phosphorus content, very low phosphorus concentration in the effluent are obtained.

For enhanced biological phosphorus removal to occur, the influent must also contain sufficient and appropriate substrates for growth of the phosphate accumulating bacteria (Rensink and Donker 1984). The lack of sufficient appropriate

substrates can be overcome by i) eliminating the primary clarifier (Mulder and Rensink 1988), ii) addition of acetic acid to the anoxic zone (Rensink and Donker 1984; Mulder and Rensink 1988), iii) addition of whole digested sludge or its supernatant to the anoxic zone (Mostert et al. 1989; Schönbergen 1989), and/or iv) coupling of appropriate industrial waste waters to a municipal wastewater treatment plant (Mostert et al. 1989). The banishment of phosphorus from detergents resulted in a decrease of the amount of substrates necessary for enhanced biological phosphorus removal (Janssen

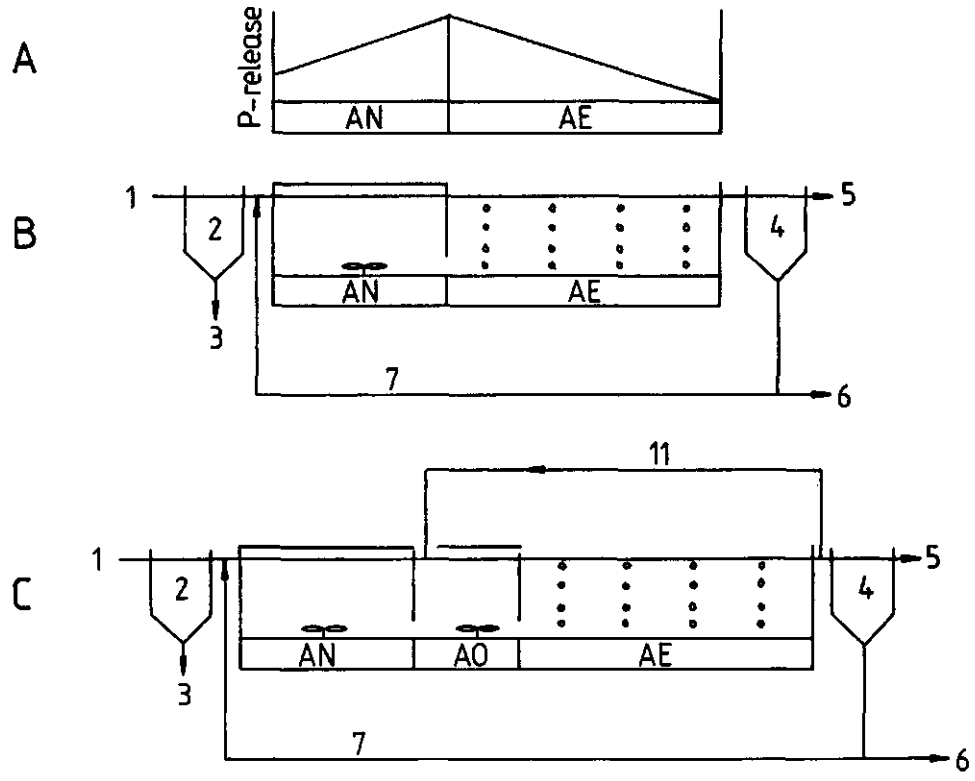

Fig. 1. Phosphorus release and uptake (A) in a wastewater treatment plant according to the "Main Stream Principle" and schematic representations of the process equipped for phosphorus removal (B) and for phosphorus and nitrogen removal (C). An: anaerobic zone/compartment without nitrate; AO: Anaerobic zone/compartment with nitrate; aerobic zone/compartment (AE); 1: sewage; 2: primary clarifier; 3: primary sludge; 4: secondary clarifier; 5: effluent; 6: surplus sludge with accumulated phosphorus; 7: return sludge. 11. internal recirculation stream of sludge/water mixture with nitrate

et al. 1990).

Treatment plants in which phosphate is only eliminated from the process by withdrawal of surplus sludge are called "main stream processes" (Fig. 1b, 1c; e.g. Schönbergen 1989).

Nitrate inhibits phosphate release. Both phosphate and nitrogen compounds can be eliminated from waste water by dividing a plant in several anoxic and oxic zones or compartments. Nitrate concentration is regulated by internal circulations (Fig. 1B; 2B). Phoredox and the University of Cape Town (UCT) processes are examples for plants removing both phosphate and nitrate compounds (e.g. Barnard 1983, Siebritz et al. 1983, Eckenfelder 1985). In stead of dividing the plant in different zones, intermittently stirring during anoxic conditions will also allow the removal of phosphate and nitrogen removal (Janssen and Rensink 1988). During quiescent periods the sludge settles and releases phosphate. Nitrate remains in the supernatant and will not influence phosphate release. During periods of stirring sludge is mixed with nitrate containing supernatant. Consequently, nitrate is converted into dinitrogen gas by denitrification. In this way, sludge is exposed alternatingly to anaerobiosis with and without nitrate. Treatment plants exhibiting excess phosphate removal plus nitrogen removal, operate generally under low organic loading rates or equivalently with high sludge ages (more than 15 days).

Treatment plants, in which phosphate is not only eliminated from the process by the withdrawal of surplus sludge, but with the aid of some side streams, are called "side stream processes" (Fig. 2). In these plants a part of the sludge is returned directly to the aeration tank, the other part is pumped to an anaerobic tank (phosphate stripping tank), in which phosphate is released (Phostripprocess, Levin et al. 1972). After the release, sludge and water are separated. Phosphate depleted sludge is returned to the aeration tank. The phosphate in the separated water is precipitated by

chemicals. For the removal of these precipitates three main processes are available, namely precipitation of phosphate by gravitation, magnetic separation and the removal with the pellet reactor. For magnetic separation magnetite and lime are added. The precipitates are separated with superconducting magnets (Van Velsen et al. 1990). In the pellet reactor sand is kept in a fluidized condition (Eggers 1988). The phosphate containing water is mixed with lime and injected into the reactor and calcium phosphate precipitates on to the sand. The phosphates produced with gravitation, magnetic separation and the pellet reactor can be reused.

In modified "side stream processes", acetic acid, fermented sludge or a part of the influent is added to the

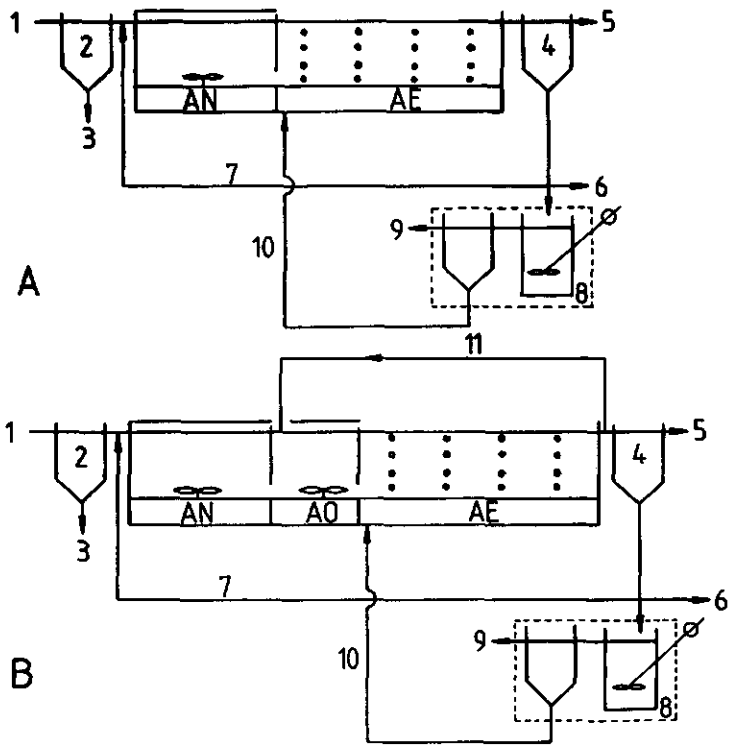

Fig. 2. Examples of wastewater treatment plants with a "Side Stream Process" configuration equipped for phosphorus removal (A) and for phosphorus and nitrogen removal (B). Symbols as in Fig. 1. 8: Anaerobic tank in which sludge releases phosphate plus an equipment for separation of the phosphorus containing effluent (9) and 'phosphorus depleted' sludge (10)

phosphate stripping tank to stimulate phosphate release (Peirano et al. 1983; De Vries and Rensink 1985). Side stream processes are well suited for low loaded activated sludge plants (Rensink et al. 1989). Successful elimination of nitrogen compounds can be obtained by incorporation of several anoxic zones into the aeration tank (Fig. 2b).

6. MICROBIOLOGY OF THE ENHANCED PHOSPHORUS REMOVAL

6.1 Discovery of the biological nature of the process

The first observations that activated sludge can remove more phosphate than is required for normal metabolic activities were made around 1960 (Srinath et al. 1959; Alarcon 1961). This phosphate removal has not always been recognized as a biological process. According to a chemical model the excess phosphate removal was due to spontaneously chemical precipitation reactions (Menar and Jenkins 1970). However, the following data suggested the biological nature of the process.

- 2,4-dinitrophenol, an uncoupler of the oxidative phosphorylation, inhibited uptake of phosphorus (Levin and Shapiro 1965; Fuhs and Chen 1975).
- Temperatures higher than 40°C and addition of certain chemicals, e.g. mercury chloride, reduced phosphate removal (Yeoman et al. 1988).
- A pH optimum of phosphorus uptake supports further the biological nature of the process (Levin and Shapiro 1965).
- Many bacteria present in enhanced phosphorus removal sludge contain polyphosphate granules (e.g. Fuhs and Chen 1975; Deinema et al. 1980; Buchan 1983; Röske et al. 1989).
- It is possible to isolate a great number of bacteria from those sludges which can accumulate high amounts of phosphorus (e.g. Fuhs and Chen 1975; Deinema et al. 1980; Buchan 1983; Streichan et al. 1990).

It has been suggested that the increased phosphate concentration during anaerobiosis may lead to chemical

precipitation of phosphates (Arvin 1983; Arvin and Kristensen 1983; Arvin and Kristensen 1984; Kerdachi and Roberts 1985; Beccari et al. 1985). The contribution of chemical phosphate precipitation to phosphate removal has been ascribed to depend on the inorganic feed composition of the sewage (Kainrath et al. 1985; Chiesa et al. 1987).

6.2 *Acinetobacter*

In general *Acinetobacter* strains are considered to be responsible for the enhanced biological phosphorus removal from waste waters (e.g. Fuhs and Chen 1975; Buchan 1983; Deinema et al. 1985). In sludge they are present in small clumps or packets and the cells are often surrounded by a thin layer of extracellular material (Deinema et al. 1985). Bacteria belonging to the genus *Acinetobacter* are obligately aerobic, Gram-negative coccoid rods. A relatively small number of strains is able to use glucose as sole carbon and energy source. Ethanol and lower fatty acids are excellent growth substrates for the majority of strains (Juni 1978). The formation of lower fatty acids in the anaerobic environment of a wastewater treatment plant with alternatingly anaerobic and aerobic conditions has been suggested to be one of the causes for the presence of acinetobacters in enhanced biological phosphorus removal (Wentzel et al. 1984; Nicholls et al. 1984).

According to Juni (1978), the genus *Acinetobacter* cannot use nitrate as an alternative electron acceptor. However, four out of the 85 *Acinetobacter* strains examined in a taxonomic study were able to reduce nitrate anaerobically in a complex medium (Bouvet and Grimont 1986). Van Groenestijn and Deinema (1985) stated that *Acinetobacter* strain 210A was able to reduce nitrate to nitrite but later research failed to confirm this observation (Van Groenestijn, personal communication). Acinetobacters, isolated by Hao and Chang (1987) were all unable to use nitrate as terminal electron acceptor. The strains, isolated by Deinema (1981) were without exceptions,

obligately aerobic. Lötter (1984) found about 50% of her strains to be able to reduce nitrate to nitrite and 8% of the strains could reduce nitrate to dinitrogen gas. A few strains were able to use glucose as carbon and energy source (Lötter et al. 1986)

Many microorganisms are able to accumulate phosphorus as a consequence of unfavourable growth conditions (Kulaev 1987). The specific conditions for excess phosphorus accumulation can be divided into two categories. Luxury phosphate uptake is the excess phosphate uptake observed when growth is inhibited by depletion of a necessary nutrient (sulphur or nitrogen) or a growth factor. Overplus phosphate uptake is observed after phosphate is added to phosphorus starved cells.

Normally, nitrogen, phosphorus or sulphur is not limited in sewage and therefore cannot cause excess phosphorus uptake (Meganck 1987). Biological phosphorus removal in excess of normal metabolic requirements during wastewater treatment has been regarded as a particular example of the overplus phenomenon, i.e. one in which the bacterial cells in the anaerobic stage are starved for phosphorus through lack of oxygen (Yeoman et al. 1988). With pure cultures of *Acinetobacter*, no effect has been observed of anaerobic conditions, proceeding normal growth (Ohtake et al. 1985; Hao and Chang 1987). Enhanced phosphate uptake by acinetobacters under microaerophilic growth conditions has been reported (Lawson and Tonhazy 1980), but most pure cultures of *Acinetobacter* accumulate phosphorus during normal logarithmic growth (Fuhs and Chen 1975; Buchan 1983; Deinema et al. 1985; Hao and Chang 1988; Van Groenestijn 1988). Phosphate uptake by enhanced biological phosphate removing sludges is therefore considered as an special example of luxury uptake (Van Groenestijn 1988).

The amount of phosphorus, which acinetobacters can contain is strain-dependent and a consequence of growth conditions (Deinema et al. 1980; Deinema 1981; Van Groenestijn and Deinema 1985; Ohtake et al. 1985; Hao and Chang 1987; Hao

and Chang 1988; Van Groenestijn 1988). Phosphorus contents on basis of dry weight of 10% (Deinema et al. 1985), 13% (Röske et al. 1988) and 13.6% (Meganck 1987) belong to the highest values reported.

Identification of acinetobacters originated from sludge removing biologically phosphate have been performed with transformation tests (Duncan et al. 1988; Beacham et al. 1990)). *A. junii*, *A. lwoffii* and *A. johnsonii* were the most commonly isolated genospecies.

6.3. Polyphosphate

Excess accumulated phosphate in microorganisms is stored as polyphosphate, usually as intracellular granules (volutin), but also other parts of the microbial cells, particularly the periplasmatic space can contain this polymer (Suresh et al. 1986; Halvorson et al. 1987; Kulaev 1987). In enhanced phosphate removal sludge and in pure cultures of *Acinetobacter* many polyphosphate granules can be observed (Deinema et al. 1980; Deinema 1981; Buchan 1983; Deinema et al. 1985; Hao and Chang, 1988; Röske et al. 1989). Anaerobically, phosphate release is coupled to a decrease in the total granular volume inside the bacterial cells. The aerobic phosphate uptake is related to an increase in granular volume (Buchan 1983; Murphy and Lötter 1986a). Periplasmatic polyphosphates are reported for *Acinetobacter lwoffii* and are possibly involved in sugar uptake and phosphorylation reactions (Suresh et al. 1986; Halvorson et al. 1987). Periplasmatic polyphosphates were detected by metachromatic spectral shifts with toluidine blue and ^{31}P-NMR spectroscopy. They disappeared under conditions of metabolic stress, while the total amount of polyphosphate did not alter (Suresh et al. 1986; Halvorson et al. 1987).

Polyphosphates can be extracted from organisms by several methods (Kulaev 1979). Accordingly they are divided into two groups: acid soluble and acid insoluble fractions. The acid

soluble fraction contains low polymeric polyphosphates (up to 20 P residues). The higher polymeric polyphosphates are present in the acid insoluble fraction.

Polyphosphates can fulfil a variety of functions in microorganisms (Kulaev and Vagabov 1983; Kulaev 1985), e.g. as a reserve of phosphate, magnesium and potassium and as an energy reserve in *Acinetobacter* (Van Groenestijn and Deinema 1985; Van Groenestijn 1988). It was found that under anaerobic conditions *Acinetobacter* can maintain a higher ATP content than closely related acinetobacters, which do not accumulate polyphosphate (Van Groenestijn 1988; Streichan and Schön 1991a). The function as an energy source allows *Acinetobacter* to survive periods when ATP cannot be formed by respiratory processes, e.g. in the case of anaerobiosis.

Based on the observation that the acid soluble fraction decreases under anoxic conditions, Mino et al. (1984) suggested that in sludge low polymeric polyphosphate functions as an energy source, and high polymeric polyphosphate serves as a phosphorus source for growth.

Magnesium and potassium seem to act as counter-ion for polyphosphate in sludge. Simultaneous uptake and release of these two ions with phosphate were observed (Miyamoto-Mills et al. 1983; Gerber and Winter 1985; Tsuzuki et al. 1987; Mostert et al. 1988; Mostert et al. 1989; Wentzel et al. 1989). Electron microscopy in combination with X-ray analysis revealed calcium as major counter ion in sludge and pure cultures of *Acinetobacter* (Buchan 1983; Heymann et al. 1989; Beacham et al. 1990). According to Kainrath et al. (1985), potassium must be considered to be the main cation of polyphosphate and the relation between calcium and polyphosphate may be an artefact. Studies with *Acinetobacter* 210a revealed a vital role for potassium in the accumulation of polyphosphate though its mode of action remained unknown (Van Groenestijn 1988). In this organism magnesium could be replaced by calcium as cation for polyphosphate as was shown by the simultaneous uptake of calcium with phosphate during

with X-ray analysis have confirmed the dependency of the elemental composition of polyphosphate granules on the magnesium/calcium ratio in the cultivation medium (Bonting et al. 1992).

6.4. Enzymes involved in polyphosphate metabolism

Kulaev (1987) has divided polyphosphate enzymes in two groups, one catalyses reactions between polyphosphates and nucleotides and the other participates in reactions without nucleotides.

Until recently, polyphosphate kinase was considered to be the most important enzyme in the synthesis and breakdown of polyphosphate. It catalyses the phosphate transfer of ATP to polyphosphate with ADP as one of the products (reaction 1).

$$(poly\text{-}P)_n + ATP \Longleftrightarrow (poly\text{-}P)_{n+1} + ADP \qquad (1)$$

With labelled ATP a very weak activity of this enzyme was reported for enhanced biological phosphate removal sludge and for pure cultures of *Acinetobacter*. The specific activity was about 1 nmol min^{-1} mg $protein^{-1}$ for the pure cultures (T'Seyen et al. 1985; 1986). Hardly any activity of this enzyme could be detected by Van Groenestijn (1988) in several *Acinetobacter* strains containing high amounts of phosphorus (up to 10% P of dry biomass). The enzyme was measured by recording ATP formation from polyphosphate + ADP in a reaction mixture containing P^1,P^5-di(adenosine-5')-pentaphosphate (AP_5A) as inhibitor of adenylate kinase. Van Groenestijn et al. (1987a, 1987b, 1988) have described an enzyme which degrades polyphosphate under anaerobic conditions in pure cultures of *Acinetobacter*. This enzyme, a polyphosphate:AMP phosphotransferase catalyses the transfer of a phosphate residue from polyphosphate to AMP (reaction 2). It was partly purified by Bonting et al. (1991). A second enzyme, adenylate kinase, converts ADP thus formed in ATP and regenerates AMP (reaction 3):

$$(poly\text{-}P)_n + AMP \dashrightarrow (poly\text{-}P)_{n-1} + ADP \qquad (2)$$

$$2\ ADP \text{ ---> } ATP + AMP \qquad (3)$$

The same enzyme system may function in phosphate removing sludges during anaerobic phosphate release (Van Groenestijn and Deinema 1987a). An increase in phosphate content of the tested sludge types correlates with the phosphotransferase activities.

A significant activity of polyphosphatase, an enzyme catalysing the hydrolysis of polyphosphate was also detected in the pure culture studied by Van Groenestijn and Deinema (1987a) (reaction 4).

$$(poly\text{-}P)_n + H_2O \text{ ---> } (poly\text{-}P)_{n-1} + P_i \qquad (4)$$

Two well-known other enzymes involved in polyphosphate metabolism, polyphosphate glucokinase and polyphosphate dependent NAD-kinase could not be detected in *Acinetobacter* strain 210A studied by Van Groenestijn (1988).

Vasiliadis et al. (1990) reported for some *Acinetobacter* strains significant activities of polyphosphate kinase and 3-phosphoglycerate kinase (18-43 and 55-92 nmol product min^{-1} mg $protein^{-1}$, respectively). However, the polyphosphate:AMP phosphotransferase activity was very low (< 10 nmol product min^{-1} mg $protein^{-1}$) or not detectable, and polyphosphate glucokinase and polyphosphate dependent NAD-kinase were absent.

6.5. Polyhydroxyalkanoates

To explain why acinetobacters increase in number in activated sludge which is alternatingly exposed to anoxic and oxic conditions, it has been suggested that acinetobacters hydrolyse polyphosphate and utilize the bond energy immediately for absorbtion and for storage of suitable substrates such as fatty acids, in particular acetate (e.g. Marais et al. 1983, Comeau et al. 1984; Murphy and Lötter, 1986b). The fatty acids produced anaerobically by other

bacteria from complex substrates are absorbed by acinetobacters and are thought to be converted into osmotic inert poly-ß-hydroxyalkanoates (PHA) with poly-ß-hydroxybutyrate (PHB) as major representative.

The conversion of substrates into PHA requires reduction equivalents in the form of $NADH_2$, which are produced either by oxidation of glycogen (Arun et al. 1988, 1989; Mino et al. 1987) or by oxidation of acetate in the tricarboxylic acid cycle (Wentzel et al. 1986). By entering the aerobic stage, PHA is oxidized and ATP can be produced, which in turn allows the replenishment of polyphosphate.

The property to sequester substrates anaerobically is thought to give acinetobacters a competitive advantage and leads to enrichment of sludge with these bacteria. Other obligately aerobic bacteria, which lack the ability to store polyphosphate under oxic conditions can only sequester the substrates aerobically.

Evidence in favour of the hypothesis that aerobic polyphosphate accumulation is related to anaerobic PHA storage is as follows

(1) Acetate stimulates phosphate release by sludge during anaerobic conditions, which was frequently accompanied with an accumulation of PHB (e.g. Rensink 1981; Arun et al. 1988; Somiya et al. 1988). Besides PHB also poly-ß-hydroxyvalerate has been reported to accumulate (Comeau et al. 1987).

(2) Bordacs and Chiesa (1989) have observed CO_2 evolution under anaerobic conditions with labelled acetate and glucose. They interpreted this result as being an argument for the anaerobic activity of the TCA cycle.

(3) In activated sludge, Buchan (1983) has observed with transmission electron microscopy the disappearance of polyphosphate granules anaerobically, which did coincide with the appearance of inclusions of "what seemed to be PHB in the same cells".

The evidence against the hypothesis that aerobic polyphosphate accumulation is related to anaerobic PHA storage is as follows:

(1) Although stimulation of phosphate release by acetate has been reported for pure cultures of *Acinetobacter* (Murphy 1986a, 1986b), several research groups could not find such a stimulation (Deinema 1981; Ohtake et al. 1985; Tsuzuki et al. 1987; Hao and Chang 1987; Hoffmeister et al. 1990).

2) Deinema et al. (1985) could only find an acetate-stimulated phosphate release in pure cultures when acetate in combination with reduced carbon compounds (e.g. isocitrate) were present. It was surmised that anaerobic PHB synthesis can solely proceed when another carbon compound is present, supplying the required reducing equivalents.

(3) With Neisser and Sudan Black staining, Lötter et al. (1986) have detected polyphosphate and PHB in aerobically grown activated sludge isolates. However, staining with Neisser or Sudan Black does not completely discriminate between granules being composed of PHB or polyphosphate (Deinema et al. 1985; Yeoman et al. 1988; Heymann et al. 1989; Anderson and Dawes 1990).

(4) Anaerobic substrate uptake by activated sludge, which was not correlated with enhanced phosphate removal, has also been reported (Fukase et al. 1985; Hao and Chang 1988; Cech and Hartman 1990).

Summarizing, we are unaware of any paper showing yet conclusively a correlation between PHB formation and enhanced biological phosphate removal.

Table 1. Bacteria other than *Acinetobacter* able to remove biologically phosphate

Organism	References
Aeromonas	Brodisch and Joyner (1983)
Pseudomonas	Brodisch and Joyner (1983)
Escherichia coli	Mostert et al. (1989)
Proteus	Mostert et al. (1989)
Klebsiella	Gersberg and Allen (1984); Mostert et al. (1989); Suresh et al. (1984)
Arthrobacter	Ohsumi et al. (1980); Shoda et al. (1980)
Gram positive bacteria	Nakamura et al. (1989); Brodisch and Joyner (1983)
Microthrix	Meganck (1987)
Moraxella	Meganck (1987); Streichan and Schön (1991b)
Xanthobacter	Streichan et al. (1990)

6.6. Other bacteria suggested being responsible for the enhanced biological phosphate removal

Many bacterial species have been described which could possibly replace *Acinetobacter* as phosphate removing organism (Table 1). Most of these bacteria were isolated from activated sludge showing excess phosphate removal, in which *Acinetobacter* could not be detected. The high number of isolates was considered as indicative for a possible involvement in phosphate removal (Brodisch and Joyner 1983; Mostert et al. 1989). A role for an *Arthrobacter* strain in enhanced biological phosphate removal was suggested due to its ability to accumulate 6.5% phosphorus on dry weight basis, although this strain was isolated from soil (Ohsumi et al. 1980; Shoda et al. 1980). 50% of the accumulated phosphorus consisted of RNA-phosphorus. Slow growing Gram-positive bacteria accumulated up to 17.8% phosphorus on total organic carbon (TOC) basis under microaerophilic conditions (Nakamura

et al. 1989). The bacteria exhibited a similar phosphate uptake and release pattern as enhanced biological phosphate removal sludge. A tentatively identified *Xanthobacter* species, two moraxellas and three acinetobacters isolated from sludges were reported to accumulate phosphorus (Streichan et al. 1990). One *Moraxella* strain was able to store polyphosphate cytoplasmatically as well as periplasmatically (Streichan and Schön 1991b). In addition, this strain could fix phosphate on the cell surface in high concentration already at pH values below 8.0. The filamentous bacteria *Microthrix* has been thought to contribute to biological phosphate removal (Meganck 1987). However, the volume of intracellular polyphosphate granules in these bacteria was not reduced during anaerobiosis, which suggest no function in excess phosphate removal (Buchan 1983). Several polyphosphate containing bacterial forms were distinguished electronmicroscopically in phosphate accumulating sludges (Röske et al. 1989). The number of *Acinetobacter*-like cells seem to depend on the influent composition and process conditions.

Several research groups have presented indirect evidence, which excluded *Acinetobacter* as only bacterial species responsible for the enhanced biological phosphate removal. For instance, ubiquinone Q-8 was found as major fraction of the respiratory quinones in sludges fed with synthetic waste water, showing anaerobic phosphate release and aerobic phosphate uptake and in sludges from a complete aerobic system (Hiraishi et al. 1989). *Acinetobacter* strains contain predominantly Q-9, an ubiquinone negligible in the sludge. With antibodies raised against acinetobacters, the amount of positive reaction in sludge was too low for explaining the phosphate removal by *Acinetobacter only* (Cloete et al. 1984; Cloete and Stein 1987, 1988a, 1988b). Similar indications were obtained with calculations based on isolation of acinetobacters from sludge by density gradient (Cloete et al. 1984; Cloete and Stein 1987, 1988a, 1988b). The suggestion was made that other bacteria or chemical precipitation of

phosphorus compounds could significantly contribute to the phosphate removal. The polyamine, diaminopropane (DAP), as biomarker for *Acinetobacter*, was used to estimate acinetobacters in activated sludges (Auling et al. 1991). Sludges originated from low organic loaded wastewater treatment plants with nitrification and denitrification had a high DAP content, while sludges from high organic loaded plants had a low DAP content. It was suggested that other bacteria than *Acinetobacter* were responsible for phosphate removal in sludges from high organic loaded plants. From the small amount of acinetobacters (12.2%) isolated from activated sludge, it was concluded that these bacteria could not be the only reason for the excellent phosphate removal of the investigated treatment plant (Kämpfer et al. 1990). However, isolation procedures for correct estimations of the number of *Acinetobacter* cells in sludge are often described as being improper due to the presence of the bacteria in clumps (Yeoman et al. 1988). In a comparison of the generally used plating technique with micromanipulation, 50-100% of the viable counts scored with micromanipulation was identified as acinetobacters, with plating only 4-18% of the viable counts were *Acinetobacter*. However, micromanipulation for isolation of acinetobacters is not completely unbiased because only clumps can be selected which are not much contaminated (Beacham et al. 1990). An evaluation of the normally used identification by the analytical profile index (API) 20E system showed that the high numbers of *Acinetobacter* in activated sludge are overestimations (Venter et al. 1989). As a consequence, it was advised to use for API tests bacteria in the logarithmic growth phase, a check of the fermentation test 24 h after the first reading and the use of a standard oxidase test.

6.7. Other bacterial groups in activated sludge flocks

In addition to excess phosphate removing bacteria several other bacterial forms and higher organisms such as protozoa can be encountered in activated sludge. Influent characteristics, operational conditions and plant design of an activated sludge plant determine the bacterial composition of a sludge population.
Important bacterial groups, which are thought to have a possible relation or can share the same process conditions with phosphorus accumulating bacteria, are: 1, acidogenic bacteria; 2, nitrifying bacteria and 3, denitrifying bacteria.

Acidogenic bacteria

Fermentation products are considered to be the most important substrates for the phosphate accumulating bacteria. From enhanced biological phosphate removal sludges many different acidogenic bacterial species have been isolated (T'Seyen 1986). In sludge the products of fermentation cannot be found. The potential uptake rate of these products by other bacteria seems to be faster than their production rate. For pure cultures of acidogenic bacteria, isolated from sludge, acetate has been found as most important fermentation product in synthetic media. Comparisons of the phosphate release by sludges with different fermentation products show in general the highest rates with acetate (e.g. Rensink 1981; Potgieter and Evans 1983; T'Seyen 1986). The amount but not the rate of the phosphate release depends on the acetate concentration initially present (Fukase et al. 1985).

Nitrifying bacteria

Most nitrifying organisms are obligate aerobic chemoautotrophic bacteria converting ammonia in two steps into nitrate. The first step is the oxidation of ammonia to nitrite, a reaction which is performed by bacteria of the

genus *Nitrosomonas*, the second step from nitrite to nitrate is conducted by the genus *Nitrobacter* (Stensel and Barnard 1992). The activity and presence of nitrifying organisms depended on the aerobic retention time, temperature, pH and other physiological factors which influence bacterial growth (Stensel and Barnard 1992). The growth rate of nitrifying organisms is slow as compared to heterotrophic bacteria. For an efficient nitrification in wastewater treatment plants the aerobic residence of the sludge has to be sufficient. Prolonged anaerobic retention times have been reported to be detrimental to nitrifying organisms (Barnard 1983).

Denitrifying bacteria

Nitrate can serve as alternative electron acceptor for some aerobic bacteria. Denitrifying organisms convert nitrate to either nitrous oxide or dinitrogen gas, depending on the strains (Tiedje 1988). Nitrite and nitrous oxide are both intermediates and often transiently detectable after the onset of anaerobiosis (Wilderer et al. 1987; Tiedje 1988). Although it is generally accepted that denitrification cannot proceed under aerobic conditions, recent findings indicate that bacteria do exist which are able to use oxygen and nitrate as electron acceptors simultaneously at dissolved oxygen concentrations at nearly air saturation (Robertson 1988). For pure cultures regulation of the synthesis of denitrifying enzymes by oxygen has been reported, but in activated sludge cycling through anaerobic and aerobic environments inhibition of the synthesis of nitrate and nitrite reductases by oxygen is not complete (Simpkin and Boyle 1988).

6.8. Effect of nitrate on phosphate release

A prerequisite for obtaining excess phosphate removal in wastewater treatment plants, is the absence of nitrate during the periods the sludge must release phosphate. In plants with long aerobic residence times, nitrate can be formed from

ammonia by nitrifying bacteria. With the return sludge, the aerobically produced nitrate enters into the anaerobic zone and prevents an efficient phosphorus release (e.g. Wentzel et al. 1984). An efficient phosphate release is necessary to obtain excess phosphorus uptake in the aerobic zone (Hascoet and Florentz 1985). The following explanations have been given for the reduction of the phosphate release by nitrate: I) phosphate precipitation due to increasing pH values under denitrifying conditions (Arvin and Kristensen 1983; Arvin 1983; Hascoet et al. 1984); II) inhibition of processes important for the phosphate release (Lötter 1984; Lötter and Van der Merwe 1987); III) the redox potential in the presence of nitrate is too high for efficient phosphate release (Rensink 1981; Peirano et al. 1983; Rensink et al. 1989); IV) competition between denitrifying and acidogenic or phosphate accumulating bacteria for the same substrate (Iwema and Meunier 1984; T'Seyen 1986; Chiesa et al. 1987; Mostert et al. 1988; Boller 1988); V) the existence of denitrifying bacteria which also accumulate large quantities of phosphate (Koch and Oldham 1985; De Vries and Rensink 1985; De Vries et al. 1985; Gerber et al. 1986; Vlekke et al. 1988; Schön and Streichan 1989; Kuba et al. 1992)

7. OUTLINE OF THE THESIS

The process of biological phosphate removal has already proven its applicability although the introductions of the process to wastewater treatment plants fails occasionally because of a lack of experience and understanding of the fundamental principles of the process. When the present investigation was started much knowledge about the physiology of pure cultures of *Acinetobacter* had been collected (Van Groenestijn 1988). However, the study with pure cultures did not give a complete insight in the complex interactions taking place in the activated sludge ecosystem. Therefore, the objective of the present research was to study activated sludges with enhanced phosphate removal.

Most studies on enhanced biological phosphate removal are conducted with activated sludges fed with undefined waste waters. To obtain a better understanding of the fundamental principles of the biological phosphate removal process, it was decided to develop a lab-scale system, which had to be simple and give possibilities to study enhanced biological phosphate removal under well defined conditions (Chapter 2). The resulting sludge types were investigated over prolonged time periods. The sludges were compared with respect to the phosphorus content, phosphate release and polyhydroxybutyric acid storage. The enrichment of different inocula with phosphate-accumulating bacteria was followed. The contribution of chemically precipitated phosphates and acid-soluble polyphosphate to phosphate removal by sludges, is described in chapter 3. Chapters 4 and 5 are dealing with the cause of the reduction of the phosphate release under denitrifying conditions. Several possible causes given for the inhibition of the phosphate release by nitrate were investigated (Chapter 4). It was found very likely that nitric oxide caused the inhibition of phosphate release. Thus nitric oxide formation by the different sludges was investigated (chapter 5). An attempt has been made to distinguish between biologically mediated nitric oxide formation and the spontaneous chemical reaction.

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Chapter 2

Biological phosphate removal by activated sludge under defined conditions

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BIOLOGICAL PHOSPHATE REMOVAL BY ACTIVATED SLUDGE UNDER DEFINED CONDITIONS

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Abstract—A simple, one reactor vessel system, called a fill-and-draw system, was developed for the study of enhanced biological phosphate removal under defined conditions. Sludge was grown in a medium with acetate and glucose as sole energy and carbon sources. The sludge was exposed to cycles with three distinct, consecutive periods; first an anaerobic period, then an aerobic period and finally a settlement period. In the period of settlement one third of the liquid was replaced with fresh medium. Sludge grown under this regime became considerably enriched with polyphosphate-accumulating bacteria. The polyphosphate content reached up to 110 mg P/g dry weight. The amount of polyphosphate in the cells during steady state depended on the acetate:glucose ratio, the nitrate and phosphate concentration in the medium. Highest phosphate accumulation was obtained with an acetate:glucose ratio of 9:1. Intracellular polyphosphate was formed during the aerobic period and was anaerobically hydrolysed and released as phosphate into the medium. In the absence of oxygen and in the presence of 2 g acetate-COD/l, 80–90% of phosphate was released by sludge containing 100 mg P/g dry weight. In the absence of acetate only 2–19% of the accumulated phosphate was excreted.

Key words—activated sludge, biological phosphate removal, phosphate, polyphosphate, wastewater treatment

INTRODUCTION

Enhanced phosphate removal from wastewater can be achieved by introducing an anaerobic zone at the front end of the conventional activated sludge process. In treatment plants modified in this way, polyphosphate-containing bacteria are enriched in the sludge (Buchan, 1983; Röske *et al.*, 1989). In general, bacteria of the genus *Acinetobacter* are considered to be responsible for the excess phosphate removal (Fuhs and Chen, 1975; Buchan, 1983; Deinema *et al.*, 1985). According to recent studies other bacterial species may also be of importance in this process, however. Nakamura *et al.* (1989) have isolated a number of Gram-positive bacteria from sludge, which are able to accumulate high amounts of phosphorus. Cloete and Stein (1988a, b) could not detect enough acinetobacters in their sludge to account for the observed phosphate removal.

For the application of enhanced biological phosphate removal several types of activated sludge plants have been designed, mainly based on empirical data and undefined sewages as influents (Eckenfelder, 1985). Studies with sludges and with pure cultures have not, until now, revealed any fully corresponding pictures about the fundamental principles of the enhanced biological phosphate removal. To increase the reliability of the process on a large scale more knowledge about these principles is required. The current investigation was undertaken to develop a simple lab-scale system for studying the enhanced biological phosphate removal under well-defined conditions and to determine some properties of the resulting sludge types. Some preliminary results of this study have been presented elsewhere (Appeldoorn and Deinema, 1987).

MATERIALS AND METHODS

Inocula

Renpho sludge originated from a pilot plant of the Department of Water Pollution Control of the Wageningen Agricultural University, The Netherlands, showing enhanced biological phosphate removal (Rensink *et al.*, 1989). The full-scale activated sludge plants Bunnik and Renkum were described previously by Janssen and Rensink (1987) and Mulder and Rensink (1987), respectively. Both plants were run as biological phosphate removal plants. Rhenen sludge originated from a carousel activated sludge plant located at Rhenen, The Netherlands, and was mainly fed with municipal wastewater. "Potato flour" sludge was sampled from a low loaded oxidation ditch located in the province of Zeeland, The Netherlands. This plant, divided into aerated and not aerated zones, treats (for 5.5 days a week) potato washing water and a starch-containing influent which had first passed through an anaerobic treatment plant. The resulting influent for the oxidation ditch had a very high COD:N ratio (700:255). Sediments were taken from Lake Loosdrecht, The Netherlands.

Media

The basic media contained (in g/l demineralized water): 0.32, NH_4Cl; 0.6, $MgSO_4 \cdot 7H_2O$; 0.07, $CaCl_2 \cdot 2H_2O$; 0.1,

EDTA and 2 ml of a trace metal solution containing (in g/l demineralized water): 1.5, $FeCl_3 \cdot 6H_2O$; 0.15, H_3BO_3; 0.03, $CuSO_4 \cdot 5H_2O$; 0.03, KI; 0.12, $MnCl_2 \cdot 4H_2O$; 0.06, $Na_2MoO_4 \cdot 2H_2O$; 0.12 $ZnSO_4 \cdot 7H_2O$; 0.15, $CoCl_2 \cdot 6H_2O$. Standard medium contained basic medium with 0.09 g K_2HPO_4, 0.05 g KH_2PO_4, 0.66 ml 96% acetic acid and 0.09 g glucose per litre. Phosphate and glucose were sterilized separately. The final pH of the medium was brought to 7.5 with 3 N NaOH.

Experimental set-up

The fill-and-draw system consisted of a 2 l. fermenter (Applicon, Schiedam, The Netherlands) containing 900 ml sludge. The set-up used was essentially the same as for continuous cultivation of microorganisms in a chemostat (Appeldoorn and Deinema, 1987). Pumps, stirring devices and air valves were controlled by digital clocks (Grässlin, Sanders-Birnie BV, Enschede, The Netherlands). The fermentor was operated in a cycle with three distinct periods, namely an anaerobic period, an aerobic period and a period allowing the sludge to settle and to replace part of the medium (Fig. 1). During the aerobic period the liquor was stirred (300 rpm) and air was bubbled through the sludge by means of a sinter stone. The oxygen capacity was always above 400 mg O_2/l/h measured in the absence of sludge (Koot, 1985). After 165 min of aerobiosis, 27 ml of the mixed liquor (medium plus sludge) was removed, the mixing and aeration stopped and the headspace of the fermentor was continuously gassed with nitrogen (technical quality). The sludge was allowed to settle for 60 min. Then, 273 ml of the supernatant was replaced by 300 ml standard medium if not otherwise stated. In total 120 min was allowed for settlement and replacement of the medium. Subsequently, while still gassing the headspace with nitrogen, the liquor was mixed (300 rpm) for 75 min. This interval is referred to as the anaerobic period and is followed again by aerobiosis. The pH was controlled automatically with 1 N H_2SO_4 and 1 N NaOH and set at 7.3. With this system and the standard medium the sludge concentration remained between 3 and 4 g dry weight/l and the mean residence time of the sludge was 8.3 days.

Determination of phosphate release

The capacity of the sludge to release phosphate was measured with sludge from the end of the aerobic period. The samples were incubated for 24 h with or without 2 g sodium acetate-COD/l in completely filled and sealed 1.5 ml disposable reaction vessels at room temperature.

When poly-β-hydroxybutyric acid (PHB) was determined, sludge from the end of the aerobic period was centrifuged, washed with demineralized water and resuspended in demineralized water. The biomass concentration was set to about 15 g dry weight/l and the suspension was gassed with a mixture of N_2:CO_2 (95.5:0.5). 5 ml of this concentrated sludge was added to a sealed 39 ml serum bottle containing 20 ml of 1.2-fold concentrated anaerobic basic medium (pH 7.5) supplemented with 4 g/l Tris–HCl and 1 g/l acetate-COD. Before adding the sludge, the bottles

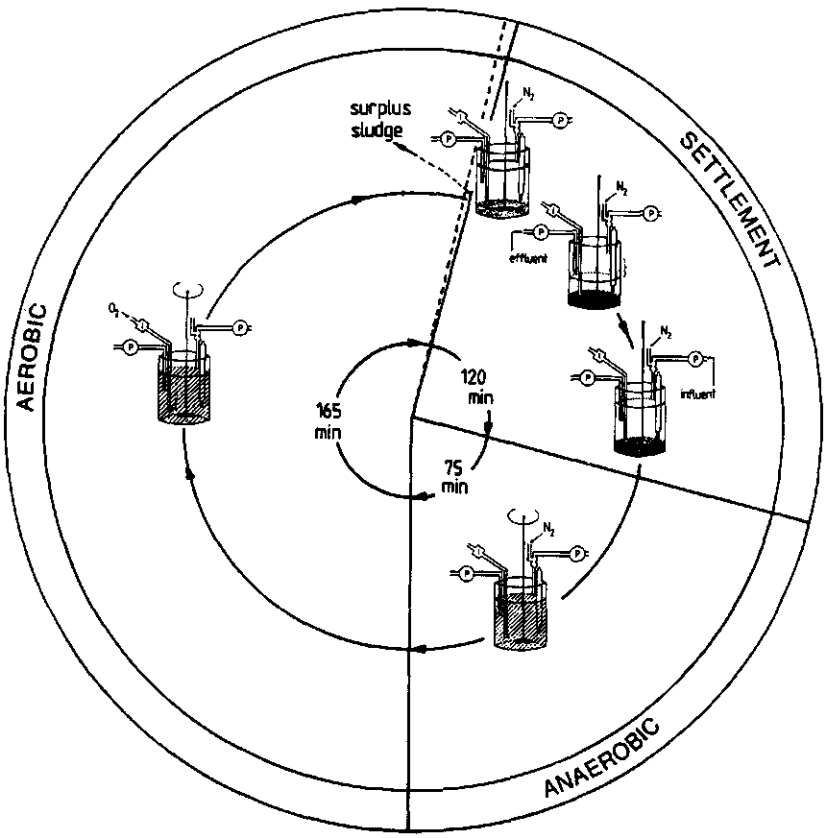

Fig. 1. Operational cycle of the fill-and-draw system.

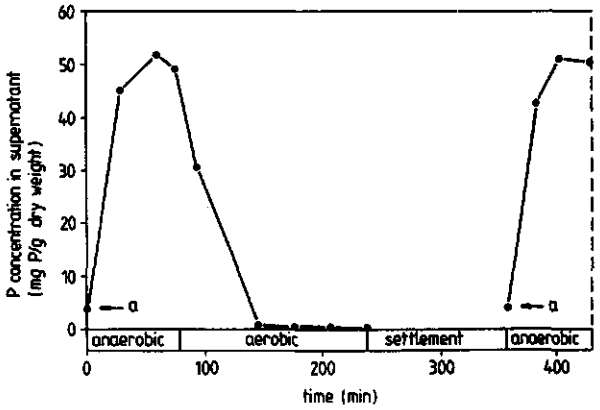

Fig. 2. Phosphate release and uptake in a fill-and-draw system fed with standard medium measured after 6 months of operation. The system was started with Renpho sludge. The sludge in the system contained about 94 mg P/g dry weight at the end of the aerobic phase. Symbols represent the mean of three independent measurements taken in the same week. a = amount of phosphorus in influent.

were flushed with the N_2:CO_2 mixture. The bottles were incubated in a thermostatted shaker at 25°C and sampled at 0, 30, 60 and 120 min.

Analytical methods

For dry weight determination the sludge was centrifuged, washed once with demineralized water and dried at 100°C for 24 h. The number of phosphorus-accumulating bacteria in the sludge was estimated microscopically after staining according to Neisser (Gurr, 1965). Orthophosphate (molybdenum–ascorbic acid method) and total phosphorus (persulphate method) were quantified according to APHA (1976). Nitrite was measured with acid sulfonilamide according to Stewart (1988). Nitrite was determined with alkaline salicilate according to Cataldo *et al.* (1975). PHB was determined after methanolysis by gas chromatographic analysis of the 3-hydroxybutyric acid methylester (Braunegg *et al.*, 1978). Sludge volume index (SVI) was determined according to Koot (1985) with the difference that only 10 ml sludge was used. Oxygen was measured with a steam sterilizable oxygen electrode (Biolafitte, Marius Instruments B.V., Den Haag, The Netherlands) connected to a potentiometric recorder (Goerz, Brown Boveri Nederland B.V., Rotterdam, The Netherlands).

Chemicals

All chemicals were of analytical grade.

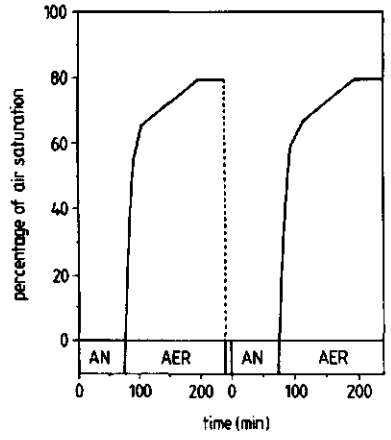

Fig. 3. Oxygen profile measured during a cycle in a fill-and-draw system fed with standard medium. During the period of sludge settlement the oxygen was not distributed evenly through the vessel. Therefore no meaningful oxygen data can be given for the settlement period.

RESULTS

Fill-and-draw system

With the fill-and-draw system, sludge with a phosphorus content up to 110 mg P/g dry weight was obtained. Anaerobically, a part of the stored phosphate was released by the sludge and taken up again under aerobic conditions (Fig. 2). At the end of the aerobic phase the phosphate concentration in the liquor was below 0.2 mg P/l. During the period of settlement hardly any phosphate was released. A typical oxygen profile during a cycle is given in Fig. 3. When sludge from a wastewater treatment plant was used as inoculum nitrification initially took place but disappeared after about 30 days. The sludge obtained in the fill-and-draw system showed an excellent settleability. The sludge volume index (SVI) was usually lower than 100 ml/g dry weight when high amounts of phosphate were accumulated.

To test the ability of the fill-and-draw system to enrich phosphate-accumulating sludge, different inocula were used (Table 1). The phosphate content of the activated sludge of the Renpho plant and a full-scale plant (Bunnik) which already possessed the capacity of enhanced biological phosphate removal could be increased by a factor of more than two after 6 weeks in the fill-and-draw system. Activated sludge from conventional municipal wastewater treatment plants (Rhenen and Renkum) could also be adapted to actively remove phosphate. However, sludge from

Table 1. Development of the phosphorus content (mg P/g dry weight) of sludge in the fill-and-draw system started with several types of inocula and fed with standard medium

Origin of inoculum	Initial phosphorus content	Final phosphorus content*
Renpho	28–35	64–87
Bunnik	25	55
Rhenen	19	52
Renkum	17	82
"Potato flour"	15	19
Lake Loosdrecht	14	18

*Determined after 6 weeks.

Table 2. Effect of feed composition on the phosphorus accumulation capacity of sludges in the fill-and-draw system. Rempho sludge served as inoculum in all experiments

Feed composition	Phosphorus content* mg P/g dry weight
Standard medium	
Basic medium + 0.86 mM P† + glucose and acetate‡	87–24
Double phosphate	
Basic medium + 1.72 mM P§ + glucose and acetate‡	110 ± 20
Nitrate	
Basic medium + 0.86 mM P† + 2.1 mM $NaNO_3$‡ + glucose and acetate‡	75 ± 17
Glucose	
Basic medium + 0.86 mM P† + glucose and acetate (COD ratio 1/1)‖	62 ± 4

*Mean value of the phosphate content during steady state conditions, measured weekly for more than 14 weeks.
†Mixture of 0.53 and 0.33 mM KH_2PO_4.
‡COD ratio 1/9, total COD 824 mg/l.
§Mixture of 1.06 and 0.66 mM KH_2PO_4.
‖Total COD was not changed.

the treatment plant of a potato flour factory and sediments from Lake Loosdrecht did not develop the ability to take up excessive amounts of phosphate within 6 weeks, despite the fact that both inocula are frequently exposed to a change between aerobic and anaerobic conditions in their natural environment. Whether a longer incubation of more than about five sludge volume changes (mean cell residence time of the sludge 8.3 days, length of the experiments 42 days) could finally lead to phosphate-accumulating sludge, was not tested. This limited series of observations indicate that the origin of the sludge is important when phosphate-accumulating sludge must be obtained within a relatively short period of time.

Feed composition

The final phosphate content of the fill-and-draw system sludge was dependent on the composition of the defined medium (Table 2). The lowest phosphate contents were obtained for sludges fed with basic medium supplied with equal amounts of glucose and acetate. COD/l (820 mg/l) and phosphate were the same as in the standard medium. The highest phosphate contents were measured in sludges fed with basic medium containing twice the amount of phosphate. In the latter case 1–3 mg P/l was left in the liquor at the end of the aerobic period. In all other cases phosphate concentration was below 0.2 mg P/l.

Enrichment and stability of the sludge

In a standard medium with twice the phosphate concentration, sludge from the Renpho plant gradually increased its phosphorus content and reached a steady state with an average maximum of 110 mg P/g dry weight after about 6 weeks [Fig. 4(A)]. At day 126 the phosphorus content decreased. At the same time nitrate, but in particular nitrite, accumulated [Fig. 4(B)]. The nitrogen oxides were formed from ammonium by nitrifiers growing on walls and baffles of the fermentor. After

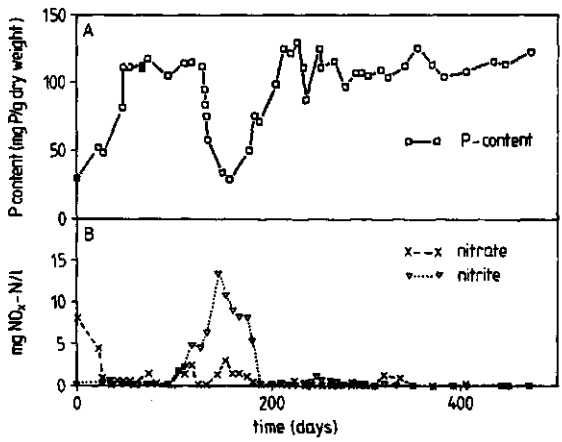

Fig. 4. (A) Phosphate accumulation (□) in a fill-and-draw system fed with standard medium plus twice the amount of phosphorus during a period of about 450 days. Phosphorus content was determined at the end of the aerobic period. (B) Nitrate (×) and nitrite (▽) concentration were determined in the liquor at the end of the aerobic period.

thorough cleaning of the fermentor on day 142, nitrate and nitrite disappeared and the phosphorus content of the sludge again reached the normal level of 110 mg P/g dry weight. The reduction of the phosphorus content of the sludge in the presence of nitrate is in accordance with the data reported in Table 2. By preventing wall growth, sludge with a high phosphate accumulation capacity could be maintained in the fill-and-draw system.

Microscopic observations revealed that during the enrichment period (first 42 days) the number of phosphate-accumulating bacteria increased up to more than 70% of the total bacterial population. Almost all bacteria in the sludge were Gram-negative (*results not shown*). The increase in phosphate-accumulating bacteria and the phosphorus content of the sludge was paralleled by an increased capacity of the sludge to release phosphate anaerobically in the presence of acetate (Fig. 5). Without acetate almost no phosphate was released. About 14 mg P/g dry weight was not exchangeable phosphorus (intersection with the regression line with the x-axis) which agrees with the average amount of organically bound phosphorus in bacterial biomass (Meganck, 1987; Meganck and Faup, 1988).

Poly-β-hydroxybutyric acid (PHB) content

Some authors hypothesized that phosphate-accumulating sludge takes up acetate anaerobically and stores it as intracellular PHB (Comeau *et al.*, 1987; Somiya *et al.*, 1988). During PHB synthesis part of the energy stored in polyphosphate is conserved in the polyester. Reducing equivalents, such as $NAD(P)H_2$ (reduced nicotinamide adenine dinucleo tide [phosphate]), are also needed for PHB synthesis. The stoichiometry is one $NAD(P)H_2$ for the conversion of 2 mol acetate into PHB. The necessary $NAD(P)H_2$ can be generated through the total oxidation of acetate via the tricarboxylic acid (TCA) cycle. The oxidation of one acetate yields 3 $NAD(P)H_2$, 1 $FADH_2$ (reduced flavine adenine dinucleotide) and 1 ATP. Through reversed electron transport the $FADH_2$ can be converted to $NAD(P)H_2$ with 1 ATP equivalent. As a result, per acetate oxidized 8 acetates can be converted to PHB. Before acetate enters the TCA cycle or is stored into PHB, acetate needs to be activated at the expense of 1 mol ATP per mol acetate if the enzyme acetate kinase is involved, or 2 mol ATP per mol acetate in case acetyl-CoA synthetase catalyses the reaction. At this moment no information is available about the pathways in which PHB is formed in enhanced biological phosphate removal bacteria. Based on the assumption that all acetates (one for the TCA-cycle and 8 for PHB-synthesis) have to be activated by acetate kinase, the synthesis of 1.2 mg of PHB requires the release of 1 mg P. If acetyl-CoA synthetase is used, only 0.6 mg PHB is produced per mg P released. In case a CoA-transferase is coupled to the 3-ketothiolase the ratio PHB formed/P released becomes 2.2 and 1.1, respectively.

During enrichment of the sludge in the fill-and-draw systems with phosphate-accumulating bacteria, phosphate release experiments were performed with the purpose of determining if the relationship between PHB storage and phosphate release does exist. In all cases the PHB content increased, while the sludge released phosphate (Table 3). However, no clear relationship was found between the amount of released phosphate and the amount of stored PHB by the sludges having different phosphorus contents. The PHB:phosphate release ratios measured after 1 h were generally in agreement with literature values (Table 3).

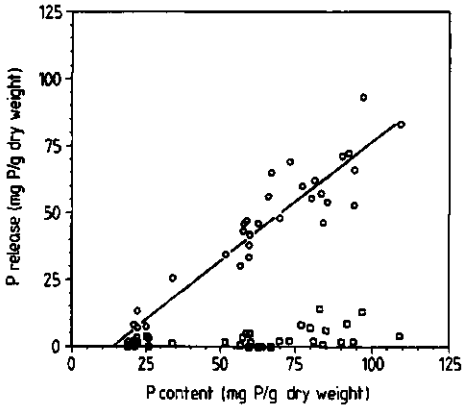

Fig. 5. Phosphate release by sludges from the fill-and-draw system after 24 h of anaerobic batch incubation with (○) and without (□) 2 g sodium acetate-COD/l. ——, Regression, $r^2 = 0.89$.

DISCUSSION

With the fill-and-draw system sludges with high phosphorus-accumulating capacities were obtained. Similar or higher phosphorus contents for sludges have been reported by others (Table 4). The so-called A/O process is the simplest way of imposing anaerobic/aerobic sequences on both wastewater and sludge. At the influent end, wastewater is mixed with recycled sludge and introduced into a plug-flow anaerobic contact zone. This anaerobic zone is generally covered, to limit air-O_2 diffusion into the mixed liquor. The main feature of the A/O process compared with other processes is the very short residence times, 0.5–1.0 h for the anaerobic zone and 1.0–3.0 h for the oxic zone (Meganck and Faup, 1988). Systems based on the Bardenpho principle and University of Cape Town (UCT) principle are multi-compartment reactors with internal sludge-recirculations. The design is made to create zones in the reactors for phosphate release, denitrification and nitrification. Wentzel *et al.* (1988, 1989) have used laboratory scale 3-stage Bardenpho systems and UCT systems to study the

Table 3. The relationship between the phosphorus release (mg P/g dry weight) and the concomitant PHB accumulation (mg hydroxybutyrate/g dry weight) after 1 h anaerobiosis with 1 g acetate-COD/l. The ratio between accumulated PHB and the P release of these experiments and some literature data (as mg hydroxybutyrate/mg P released) is given

Biomass	P content*	P release*	PHB†	PHB/P release ratio
Renpho	29	4	5	1.3
	30	2	6	3.0
	38	8	3	0.4
Fill-and-draw	44	18	63	3.5
	60	36	17	0.5
	60	33	48	1.5
	61	52	59	1.1
	101	53	70	1.3
	116	67	41	0.6
Literature data:				
Mino *et al.* (1987)				0.9–2.2
Comeau *et al.* (1987)				0.9
Arun *et al.* (1988)				0.6–1.7
Theoretical value				
(1 mol acetate oxidized	With acetate kinase			1.2
via the TCA	With acetate kinase and CoA-transferase			2.2
cycle; 8 mol acetate	With acetyl-CoA synthetase			0.6
converted into PHB)	With acetyl-CoA synthetase and CoA-transferase			1.1

*Mg P/g weight.
†Mg PHB/g dry weight.

effect of a synthetic medium on the development of enhanced biological phosphate removal. The feed contained, besides inorganic salts, acetate as sole organic substrate. The laboratory scale pilot plant described by Hascoet *et al.* (1984) consisted of a single compartment reactor and a secondary clarifier. Anaerobic and aerobic phases in the reactor were controlled by a programming clock. During anaerobiosis the reactor was fed with a synthetic substrate based on meat extract. The experimental unit of Fukase *et al.* (1985) consisted of two anaerobic vessels, the same number of aerobic vessels and a settling vessel. Their synthetic medium was composed of acetate, peptone and yeast extract plus inorganic salts. For all synthetic wastewaters, the maximum phosphorus content of the sludges was above 100 mg P/g dry weight (Table 4). The highest phosphorus-accumulating values for sludges on lab-scale systems reported by Wentzel *et al.* (1988, 1989) are 180 mg P/g dry weight which means that more than half of the sludge dry weight was composed of phosphate in its monomeric or polymeric form. In comparison, the highest measured phosphorus contents for pure cultures of *Acinetobacter* were 100 mg P/g dry weight (Deinema *et al.*, 1985) and 136 mg P/g dry weight (Meganck, 1987), respectively.

Enhanced biological phosphate removal is dependent on the exposure of the activated sludge to short-chain organic acids in the anaerobic stage (Toerien *et al.*, 1990). Based on the COD composition and loading rates, domestic wastewater will, on average, contain or produce less of these specific compounds than plants fed artificially with the right substrate mixture. As a consequence, activated sludge grown on domestic wastewater will also contain less phosphorus-accumulating bacteria. In systems fed with domestic wastewater the highest phosphorus concentration reported was 68 mg P/g dry weight (Rensink *et al.*, 1979). This result was obtained under high sludge loadings. Normally, the phosphorus content of sludges of enhanced biological phosphate removal wastewater treatment plants do not exceed 40 mg P/g dry weight.

A possible important involvement of Gram-positive bacteria in the enhanced phosphate removal process as suggested by Nakamura *et al.* (1989) could

Table 4. Maximum phosphorus contents reported for several enhanced biological phosphate removal systems

System	Feed*	Maximum P content (mg P/g dry weight)	References
Pilot plant high loading conditions	Domestic sewage	68	Rensink *et al.* (1979)
A/O process	Domestic sewage	60	Manning and Irvine (1985)
Bardenpho	Domestic sewage	40–60	Manning and Irvine (1985)
Renpho	Domestic sewage	35	This paper
Single reactor with settlement tank	Synthetic wastewater (meat extract)	110	Hascoet *et al.* (1984)
Artificially designed system with several compartments	Synthetic wastewater (acetate/pepton/yeast)	110	Fukase *et al.* (1985)
3-stage Bardenpho lab-scale	Synthetic wastewater (acetate)	180	Wentzel *et al.* (1988, 1989)
University of Cape Town lab-scale	Synthetic wastewater (acetate)	180	Wentzel *et al.* (19888, 1989)
Fill-and-draw	Synthetic wastewater (acetate/glucose)	110	This paper

*In parentheses carbon sources of the synthetic wastewaters.

be excluded for the sludge in the fill-and-draw system. Microscopically, almost all bacteria in the sludge were Neisser-positive as well as Gram-negative. This finding is in agreement with Li and Ganczarczyk (1990), who also observed mainly Gram-negative bacteria in sludge exhibiting enhanced biological phosphate removal.

A relation between PHB formation and phosphate release during anaerobic conditions has been suggested by several research groups (e.g. Comeau *et al.*, 1987; Somiya *et al.*, 1988). It is thought that ATP from polyphosphate hydrolysis is used anaerobically for PHB synthesis. PHB would so act as a storage material of fatty acids which are abundant under these conditions. Much has been written about the coupling of PHB formation and polyphosphate hydrolysis (e.g. Mino *et al.*, 1987; Comeau *et al.*, 1987; Arun *et al.*, 1988) but more than indirect evidence has not yet been presented. Studies in which tracers are followed into specific organisms in the sludge are needed to clarify this phenomenon which is debated by many authors (Deinema *et al.*, 1985; Fukase *et al.*, 1985; Ohtake *et al.*, 1985; Tsuzuki *et al.*, 1987; Hao and Chang, 1987). Attempts to find a relationship in fill-and-draw system sludges between PHB accumulation and phosphate release or phosphorus content were not successful. The observed PHB:P release ratios were generally in agreement with the variations for these ratios reported in the literature (Table 3).

This paper shows that with a relatively simple system, sludge enriched with phosphate-accumulating bacteria can be obtained. This sludge can be maintained over a prolonged period of time. Due to its simplicity, the fill-and-draw system is well suited for the acquisition of a better understanding of the enhanced biological phosphate removal process on laboratory scale.

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Chapter 3

Contribution of precipitated phosphates and acid-soluble polyphosphate to enhanced biological phosphate removal

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CONTRIBUTION OF PRECIPITATED PHOSPHATES AND ACID-SOLUBLE POLYPHOSPHATE TO ENHANCED BIOLOGICAL PHOSPHATE REMOVAL

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Abstract—The amount of precipitated metal phosphates and the acid-soluble polyphosphate concentration were measured in several sludges and pure cultures of *Acinetobacter* strains. The percentage of metal phosphates of the total phosphorus content of the sludges varied between negligible and about 80%. Increasing ability to release phosphate anaerobically was frequently parallelled by a decreasing contribution of precipitated metal phosphates to the total phosphorus concentration in 10 different sludges. Sludges from fill and draw systems with over 100 mg P/g dry wt, were almost completely devoid of these precipitates. The same was true for polyphosphate-accumulating cultures of *Acinetobacter*. Besides high molecular weight polyphosphate, the sludges also contained low polymeric polyphosphates (LPP), ranging from 0 to 50% of the total phosphate content. The LPP fraction in polyphosphate-accumulating cultures of *Acinetobacter* strains was between 3 and 23% of their total phosphate content. Sludges developed in the fill and draw systems could release more than 50% of the accumulated phosphate within 60 min. About 66% of this phosphate originated from LPP. Pure cultures of *Acinetobacter* strains released less than 1 mg P/g dry wt in the same time. Reduction of phosphate release by an increase of the pH in the medium as a result of denitrification could only partly be explained by formation of precipitated phosphates.

Key words—*Acinetobacter* spp, biological phosphorus removal, polyphosphate, metal phosphate, phosphate, wastewater treatment

INTRODUCTION

Incorporation of an anaerobic phase at the influent end of the activated sludge process can lead to a higher phosphate removal as compared to the completely aerobic process. The anaerobic phase is thought to select sludges enriched with polyphosphate-accumulating bacteria of the genus *Acinetobacter* (Toerien *et al.*, 1990). In these sludges, excessive amounts of phosphate are taken up aerobically and released in absence of oxygen. It has been suggested that the increased phosphate concentration in the medium during anaerobiosis may lead to precipitation of phosphates and therefore reduce the observed phosphate release (Arvin, 1983; Arvin and Kristensen, 1983; Beccari *et al.*, 1985; Miya *et al.*, 1987). Reduced phosphate release under denitrifying conditions has also been explained in terms of precipitation of phosphates (Arvin, 1983); denitrification increases the pH in the microenvironment of the sludge flocs which may enhance phosphate precipitation by calcium, magnesium or iron.

To distinguish between biologically-stored phosphates and chemically-precipitated phosphates, treatment of activated sludges or pure cultures with perchloric acid (PCA) or trichloroacetic acid has been used (e.g. Carberry and Tenney, 1973; Fuhs and Chen, 1975; Mino *et al.*, 1984; Ohtake *et al.*, 1985; Murphy and Lötter, 1986). Calcium, magnesium and iron phosphates are dissolved by these acids. Besides the phosphate salts, intracellular low polymeric polyphosphates (LPP), with a chain length of up to 20 residues, are also solubilized. The higher polymeric polyphosphates (HPP) are not extracted by cold acids (Kulaev, 1979).

This investigation was undertaken to determine the contribution of precipitated metal phosphates and acid-soluble polyphosphates for enhanced biological phosphate removal. Pure cultures of polyphosphate-accumulating *Acinetobacter* were used in this study as well.

MATERIALS AND METHODS

Organisms

Acinetobacter strains 210A, 1, B8 and P were isolated from activated sludge by the method described by Deinema *et al.* (1980). These organisms were maintained on yeast extract agar slants (5 g glucose, 2.5 g yeast extract and 12 g agar per litre tap water, pH 7.0), and subcultured every 2 months and stored at 4°C.

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Abbreviations used: LPP, low polymeric polyphosphates; HPP, high polymeric polyphosphates; PCA, perchloric acid.

Sludges

Fill and draw systems were run as described by Appeldoorn *et al.* (1992). The synthetic media used were standard medium, standard medium supplemented with 2.1 mM nitrate or standard medium with twice the amount of phosphate (Appeldoorn *et al.*, 1992).

Sludges mainly fed with municipal wastewaters originated from: Coevorden, a Schreiber type activated sludge plant operating with alternating anaerobiosis and aerobiosis to obtain maximum ammonium and nitrate elimination. Oosterwolde, a carrousel activated sludge plant; its aeration capacity was insufficient under high loading conditions. Kortenoord, an oxidation ditch with inadequate aeration devices. Vlagtwedde, an oxidation ditch discontinuously fed with influent stored for 5 h in the sewer. In this plant aeration was temporarily interrupted during feed. These activated sludge installations were selected based on their phosphate removal efficiencies described in the annual reports of all wastewater treatment plants in The Netherlands (unpublished results of Hennink and Rensink). Sludges of these plants released 4–10 mg P/g dry wt with 40–50 mg acetate-COD/g dry wt within 3 h anaerobiosis (unpublished results of Hennink and Rensink). Sludges from the plants Beilen, Bunnik, Bunschoten, Rhenen and Zeeland and from the pilot plants Renpho and Spanjers were also included in this study. Beilen is a trickling filter plant receiving mostly discharges of a dairy factory with extremely high pH values in the influents. Rhenen (treating municipal wastewater) and Zeeland (treating wastewater of a potato flour factory) have been characterized by Appeldoorn *et al.* (1992). Bunnik and Bunschoten have been reported to show enhanced biological phosphate removal (Janssen and Rensink, 1987). Renpho sludge originated from an enhanced biological phosphate removal pilot plant of the Department of Wastewater Pollution Control of the Wageningen Agricultural University, The Netherlands. The pilot plant operated according to the Renpho principle (Rensink *et al.*, 1989). Spanjers is a pilot scale plant of the same department with optimal aeration devices (Spanjers and Klapwijk, 1986).

Sludge samples were taken at the end of the aerobic period. They were immediately centrifuged for orthophosphate determination in the supernatant. In some cases (Zeeland, Bunnik, Bunschoten) orthophosphate determination was done after aeration for several hours at the laboratory. Sludge samples were kept on ice during transport from the treatment plant to the laboratory.

Cultivation of acinetobacters

The strains were grown in shaken Erlenmeyer flasks and harvested in the logarithmic phase. The medium was essentially the same as described by Van Groenestijn *et al.* (1987) except the trace mineral salts solution which was from Appeldoorn *et al.* (1992). Strain 210A was cultivated in medium with 21 mM butyrate at 15°C or with 42 mM acetate at 15, 20 and 27°C. Strain 1 was cultivated in acetate medium at the same temperatures, strain B8 and P were cultivated in acetate medium at 15°C. Strain 210A was also cultivated in a 2 l. bioreactor (Applikon, Schiedam, The Netherlands) with automatically controlled pH (7.0) and temperature (20°C). Tris–HCl buffer was omitted from the acetate medium used in the bioreactor. For phosphate release experiments and the determination of the phosphate fractions, the bacteria were concentrated to about 5 g/l.

Determination of phosphate release

The capacity of the sludge to release phosphate was measured after incubation for 24 h at room temperature with or without 33 mM sodium acetate in completely filled and sealed 1.5 ml disposable vessels.

When the effect of nitrate on the phosphate release was determined, sludge from the end aerobic period was centrifuged, washed with demineralized water and resuspended in demineralized water. The biomass concentration was set to about 15 g dry wt/l and the suspension was gassed with a mixture of 99.5% N_2 and 0.5% CO_2. 5 ml of the sludge suspension was added to sealed 39 ml serum bottles containing 20 ml anaerobic release medium (pH 7.5) containing per litre demineralized water: 0.38 g NH_4Cl, 0.7 g $MgSO_4 \cdot 7H_2O$, 0.08 g $CaCl_2 \cdot H_2O$, 0.12 g EDTA, 4.8 g Tris–HCl, 1.2 g acetate-COD and 2.4 ml of a mineral salts solution (Appeldoorn *et al.*, 1992). The bottles were flushed with a mixture of 99.5% N_2 and 0.5% CO_2 before adding the sludge. After addition of the sludge, the bottles with or without 3.6 mM sodium nitrate were immediately incubated in a thermostated shaker at 25°C. Tris–HCl buffer was omitted in experiments with non-buffered medium.

To determine the efficiency of the PCA method to dissolve precipitated metal phosphates, experiments were carried out in anaerobic serum bottles under an atmosphere of N_2:CO_2 (99.5:0.5). The bottles contained 20 ml release medium supplemented with 3.3 mM phosphate and 6 mM of either calcium, iron (II) or iron (III) chloride at a final pH of 7.5, 5 ml concentrated pasteurized fill and draw system sludge up to a dry weight of about 3 g/l, or 5 ml demineralized water. After shaking for 1 and 2 h at 25°C, samples were taken from the bottles. The amount of precipitated phosphates in the samples was measured after treatment with PCA, as described in the analytical methods.

Analytical methods

For dry weight determination, the sludge or microbial biomass was centrifuged, washed once with demineralized water and dried at 100°C for 24 h.

Orthophosphate (ascorbic acid method) and total phosphorus (persulphate method) were quantified according to APHA (1976). Total phosphorus determinations were done in triplicate.

Analysis of the different phosphorus fractions was carried out according to Harold (1960). Sludge and microbial biomass was first washed with demineralized water. The pellet was resuspended and stored for 20 min in cold 0.5 N PCA in the refrigerator. The suspension was centrifuged and extracted once more in the same way. The cold PCA extracts were pooled and examined for orthophosphate (metal phosphate) and for total phosphate with the persulphate method. The LPP fraction was assumed to be the total phosphate minus orthophosphate. The pellet was further extracted with ethanol and with ethanol + ether (3:1, v/v) to remove lipids and phospholipids. Extraction of the residue with hot 0.5 N PCA (70°C) for 15 min removed HPP and nucleic acids from the cells. To assure complete removal this extraction was repeated. If not indicated otherwise the volumes of PCA, ethanol and ethanol/ether were the same as the original sludge or microbial biomass suspensions.

Chemicals

All chemicals used were of the highest analytical grade available.

RESULTS

Precipitated phosphates

Iron (II), iron (III) or calcium phosphates precipitated in the release medium were well dissolved by PCA, both in the presence and absence of pasteurized fill and draw system sludges containing at least 60 mg P/g dry wt (Table 1).

When the method was applied to several sludge types, more than 80% of the totally measured precipitated phosphates was dissolved during the first cold PCA treatment. Larger volumes of cold PCA per volume sludge did not increase significantly the

Table 1. Recovery of phosphate from iron (III), iron (II) and calcium salts after treatment with 0.5 N perchloric acid

Additions to the release medium*		(%)†
6 mM $FeCl_3$		104
6 mM $FeCl_3$	+3 g/l pasteurized sludge from a fill and draw system	80
6 mM $FeCl_2$		92
6 mM $FeCl_2$	+3 g/l pasteurized sludge from a fill and draw system	103
6 mM $CaCl_2$		100
6 mM $CaCl_2$	+3 g/l pasteurized sludge from a fill and draw system	89

*3.3 mM KH_2PO_4 was added in all experiments.
†Values are means of 2 independent measurements.

extraction efficiency of precipitated phosphates (Table 2). Except for two sludges (Spanjers and Beilen) all other sludges released phosphate after incubation for 24 h with 2 g acetate-COD/l. This is an indication for the capacity of these sludges for enhanced biological phosphate removal (Toerien *et al.*, 1990; Keurentjes and Iwema, 1987). Some sludges (Beilen, Coevorden, Vlagtwedde) contained appreciable amounts of chemically-precipitated phosphates, which may contribute 30–85% of the phosphate removal in these wastewater treatment plants.

In fill and draw system sludges with high phosphorus contents the contribution of the precipitated phosphates to the phosphate removal was almost always lower than 3 mg P/g dry weight (Table 2, Fig. 1). A considerable portion of the phosphorus was present in this sludge as LPP (Fig. 1, Table 3). When fill and draw sludge was kept at pH 9.1 about 60% of the phosphorus was present as acid-soluble orthophosphate (Table 2).

Precipitated phosphates in pure cultures of *Acinetobacter* were below 3 mg P/g dry wt.

Denitrification and precipitation of phosphates

Renpho sludge released phosphate at a rate of 4–8 mg P/g dry wt/h in the release medium. In the presence of nitrate this rate was reduced by more than 40%. No additional phosphate precipitation could be measured. In media with nitrate but lacking Tris–HCl buffer a slight increase in precipitated phosphates was observed (Fig. 2). The pH increased to 9.2 after 240 min. Without nitrate no additional phosphate precipitated and the final pH was 8 after 240 min of anaerobic incubation.

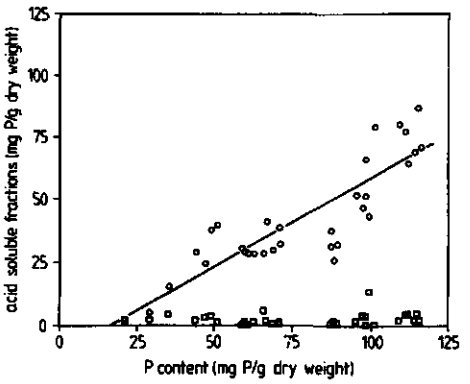

Fig. 1. Relationships between total P content, LPP content (○) and precipitated phosphates (□) in fill and draw system sludges. ——, Regression, $r^2 = 0.75$. Cells devoid of the polyphosphate contain 16.8 mg P/g dry wt (intercept of regression line with x-axis). The data were obtained during start-up of fill and draw systems with sludge from the Renpho pilot plant as inoculum, containing about 30 mg P/g dry wt (Appeldoorn *et al.*, 1992).

Organic phosphates

About 80% of the total amount of phosphorus in *Acinetobacter* pure cultures or in fill and draw system sludges could be extracted with cold and hot PCA. In the hot PCA fraction 33% of the total amount of phosphorus was found as orthophosphate indicating that a significant part of the organic phosphates and/or polyphosphate was hydrolysed by this treatment. This indicates that the hot PCA treatment is probably too harsh for estimating the amount of phosphorus in HPP and nucleic acids.

The amount of nucleotide phosphates were estimated in the cold PCA fraction by the addition of 0.2 g norit (Harold, 1960). Norit removes organic phosphorus compounds from acid solutions, leaving inorganic and polyphosphate behind. Treatment with

Table 2. Contribution of precipitated phosphates to the phosphorus content of several sludges

Sludge	Total P content*	P release* detected after 24 h with 31 mM acetate	Precipitated P* PCA/sludge ratio 1:1	2:1	4:1	6:1
Spanjers	16	1	5	6	6	5
Bunnik	24	5	ND†	7	ND	ND
Kortenoord	26	7	7	8	8	8
Coevorden	27	5	10	10	11	12
Bunschoten	27	9	ND	9	ND	ND
Oosterwolde	29	8	5	6	6	7
Vlagtwedde	30	6	9	10	13	15
Beilen	43	1	34	34	38	41
Renpho	48	15	5	5	5	7
Fill and draw	87	59	ND	1	ND	ND
Fill and draw pH = 9.1	87	13	ND	60	ND	ND

*mg P/g dry wt, measurements have been performed in duplicate.
†Not determined.

Table 3. LPP content of sludge and of pure cultures of *Acinetobacter* spp

Sludge	P content* (mg P/g dry wt)	P release†	LPP (% of the P content)
Group 1‡	15–19	0–2	1–7
Group 2‡	27–30	5–6	0–3
Group 3‡	26–29	6–10	12–14
Group 4‡	25–28	5–10	24–26
Renpho	28–35	11–14	22–29
Fill and draw	47–87	29–69	41–50
Acinetobacter strain 1	52–62	39–43	20–23
Acinetobacter strain 210A	39–88	21–60	3–20
Acinetobacter strain B8	47	ND	9
Acinetobacter strain P	56	46	15

*The highest and lowest values of the P content within the groups plus the variation of these values for the sludges or the variations within the acinetobacter strains are given.

†P release (mg P/g dry wt) after 24 h incubation with 31 mM sodium acetate. ND = not determined.

‡Sludges are arranged according to P release. Group 1: Spanjers/Zeeland/Rhenen; Group 2: Coevorden/Vlagtwedde; Group 3: Oosterwolde/Kortenoord; Group 4: Bunnik/Bunschoten.

norit reduced the total amount of phosphorus in the cold PCA fraction extracted from Renpho sludge or *Acinetobacter* 210A with 2–3 mg P/g dry wt.

Fructose phosphates were estimated in the sludge of Bunnik and Bunschoten and in the sludge of the fill and draw systems. Values ranging from 0 to 1 mg P/g dry wt were found using the method described by Langen and Liss (1958).

Low polymeric polyphosphates

All LPP could be removed from the biomass by repeating the extraction with the cold PCA. The first PCA fraction usually already contained 80% of the total amount of LPP. Polyphosphate hydrolysis during the orthophosphate analysis has been reported by De Haas *et al.* (1990). This can result in overestimations of the amount of precipitated phosphate. The constant low amounts of precipitated phosphate in the fill and draw system sludges shows that here LPP hydrolysis does not occur to a significant extent (Fig. 1, Table 2). Table 3 reports the quantities of LPP found in a variety of sludges and pure cultures. There is a tendency that sludges with higher amounts of LPP also released more phosphate anaerobically. The total phosphate content and the quantity of LPP in the fill and draw system sludges correlated positively (Fig. 1). LPP had almost completely disappeared from this sludge after 2 h of anaerobiosis (Fig. 3). About 66% of the excreted phosphate could be explained by the degradation of LPP. Apparently other cellular phosphorus fractions are degraded as well. The nature of these fractions was not examined.

In batch grown cultures of *Acinetobacter*, the LPP fraction was generally lower than 23% of the total phosphorus content (Table 3). This percentage is much lower than the percentage in fill and draw system sludges. In *Acinetobacter* 210A grown in a chemostat at dilution rates between 0.06 and 0.3 h^{-1} the LPP fraction remained low, but increased steadily with the dilution rate from 3 to 8 mg P/g dry wt. This is 10% or less than the total phosphorus content reported by Deinema *et al.* (1985) and Van Groenestijn *et al.* (1989). All pure cultures released phosphate at much lower rates than the tested phosphate-accumulating sludges. The fill and draw system

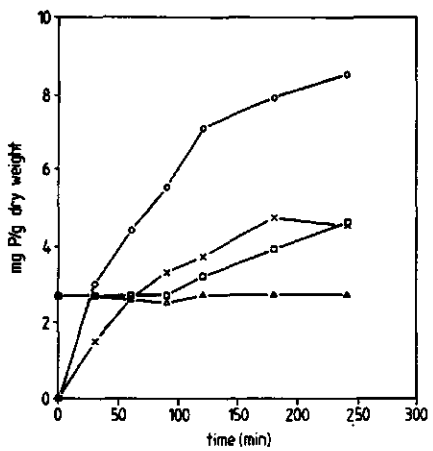

Fig. 2. Phosphate release by Renpho sludge in a medium lacking Tris–HCl buffer without nitrate (○), with 3.6 mM nitrate (×) and precipitated phosphates without nitrate (△) and with 3.6 mM nitrate (□).

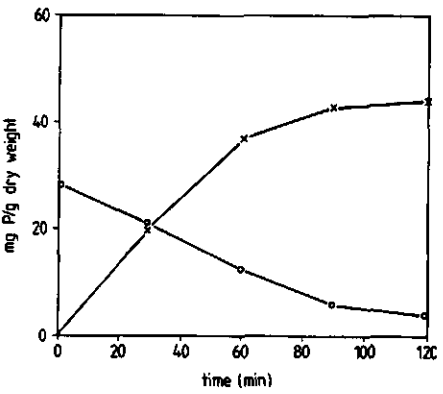

Fig. 3. Phosphate release (×) and LPP degradation (○) during anaerobiosis by a fill and draw system sludge containing 60 mg P/g dry wt.

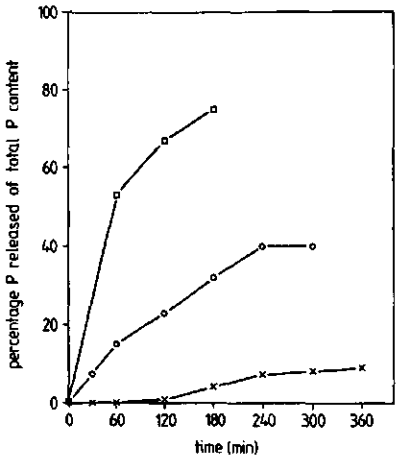

Fig. 4. Percentage of phosphorus of the phosphorus content released by fill and draw system sludge (□), Renpho sludge (○) and *Acinetobacter* strain 210A grown at 15°C (×). At the start of the experiments the phosphorus content of the biomass was 81, 28 and 65 mg P/g dry wt, respectively.

sludge excreted more than 50% of its accumulated phosphorus within 1 h, whereas about 9% of the phosphorus accumulated by *Acinetobacter* 210A appeared in the medium after 6 h anaerobiosis. Renpho sludge excreted phosphate at a lower rate as compared to the sludge from the fill and draw system (Fig. 4). This may be explained by the lower phosphorus content of the Renpho sludge.

DISCUSSION

Chemically-precipitated phosphate in the presence of activated sludge can be efficiently dissolved by the cold PCA method. This conclusion is in line with observations of several other research groups (Mino and Matsuo, 1985; Kerdachi and Healy, 1987). The chemically-precipitated phosphates can contribute to the phosphate removal by sludges in wastewater treatment plants. The sludges examined in this study could roughly be divided into three groups. Sludge type 1 (Spanjers, Zeeland, Rhenen, Beilen) does not release orthophosphate under anaerobic conditions and precipitated phosphates can, to a variable extent, contribute to the phosphate content. Sludge type 2 contains both polyphosphate and orthophosphate precipitates (Oosterwolde, Kortenoord, Bunnik, Bunschoten, Renpho). In sludge type 3, represented by the fill and draw system sludge, only limited amounts of precipitated phosphates are present. The major part of the phosphate is accumulated in bacterial cells.

Several research groups have obtained sludges with high amounts of phosphorus (Hascoet *et al.*, 1984; Fukase *et al.*, 1985; Wentzel *et al.*, 1988, 1989). In fill and draw system sludges and in pure cultures of *Acinetobacter* with high amounts of phosphorus, hardly any precipitated phosphate was detected. This confirms that bacteria and sludge can accumulate over 100 mg P/g dry wt intracellularly and that this high amount is not a result of chemical precipitation. The data presented in this paper also show very clearly that an increase of the pH during denitrification does not fully explain the reduced phosphate release in the presence of nitrate.

In sludges from plants with enhanced biological phosphate removal, HPP has been reported as the major polyphosphate fraction responsible for phosphate release and immobilization (Fuhs and Chen, 1975; Hiraishi *et al.*, 1989). Murphy and Lötter (1986) found that the acid-soluble as well as the acid-insoluble polyphosphates both contribute to the phosphate release process. In our experiments, sludges showing good phosphate release contained in general more LPP than sludges which hardly released any phosphate. This is in accordance with Mino *et al.* (1984, 1985), who also found large amounts of LPP in phosphate-accumulating sludges and calculated that the phosphate released originated mainly from the hydrolysis of LPP.

Pure cultures of *Acinetobacter* spp possessed much less LPP than fill and draw system sludges. Low contributions of LPP to the phosphate content of several *Acinetobacter* strains were also reported by Ohtake *et al.* (1985). The formation of only small LPP concentrations by pure cultures of *Acinetobacter* was not expected since enhanced biological phosphate removal sludge is thought to be composed of large numbers of acinetobacters. Variations of growth conditions have been shown to affect not only the amount of polyphosphate formed but also the chain length in *Candida* species (Núñez and Calleri, 1989). However, in *Acinetobacter* strain 210A different growth rates imposed in a chemostat did not markedly influence the LPP concentration. One may speculate that the strains tested were not representative of the acinetobacters in sludge or that in addition to acinetobacters, polyphosphate-accumulating sludge contains other bacteria synthesizing large amounts of LPP. The possibility that bacteria other than *Acinetobacter* may be responsible for the enhanced biological phosphate removal has been suggested by several research groups (e.g. Gersberg and Allen, 1984; Suresh *et al.*, 1984; Röske *et al.*, 1989; Nakamura *et al.*, 1989).

A second difference between phosphate-accumulating sludge and pure cultures of phosphate-accumulating *Acinetobacter* strains is the relatively slow phosphate release by these strains. A relatively slow release by pure cultures has also been reported by Deinema *et al.* (1985), Ohtake *et al.* (1985) and Nakamura *et al.* (1989). The slower phosphate release may be related to the much lower LPP content of the pure cultures. It may be speculated that LPP is a more appropriate substrate for polyphosphate-degrading enzymes resulting in a faster phosphate

release as compared with the phosphate release by bacteria containing HPP as the major polyphosphate fraction.

CONCLUSION

From this study it has become clear that precipitated phosphates may play a role in the phosphate removal by sludges in wastewater treatment plants. However, its contribution depends on sludge type, wastewater composition, treatment design, etc. In sludges cultured in the fill and draw systems, phosphate precipitates add only marginally to the phosphate removal capacity.

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Chapter 4

The effect of nitrate on the phosphate release of sludge removing biologically phosphate

THE EFFECT OF NITRATE ON THE PHOSPHATE RELEASE OF SLUDGE REMOVING BIOLOGICALLY PHOSPHATE

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ABSTRACT

Sludge of a wastewater treatment plant designed to remove biologically phosphate, released phosphate when incubated under anaerobic conditions in the presence of acetate. The initial phosphate release rate was reduced by addition of nitrate. In the presence of 16 mM acetate, maximum inhibition of 40 to 50% occurred at a nitrate concentration of 3.6 mM. Increasing the amount of acetate 10 times above the theoretical amount needed for complete denitrification, did not overcome the effect of nitrate. After depletion of nitrate, the phosphate release rate recovered slightly. In the presence of KCN and azide, inhibitors of denitrification, nitrate did not inhibit phosphate release. After completion of phosphate release and with sufficient acetate present, phosphate was taken up after addition of 3.6 mM nitrate. This phosphate uptake was too small to explain the reduction of the initial phosphate release rate, when nitrate was added at the start of the experiments. Nitric oxide added at a concentration of 0.3 mM in the liquid phase inhibited the rate of phosphate release by 40%, whereas 3.6 mM nitrous oxide had no negative effect. Nitrite showed about the same effect as nitrate. The inhibitory effect of nitric oxide on the phosphate release was slightly reduced by addition of 5 mM KCN. The data presented here show that the negative effect of nitrate on the phosphate removal can be caused by its conversion to nitric oxide.

INTRODUCTION

The increased discharge of nitrogen and phosphorus compounds is one of the factors responsible for the eutrophication of surface waters. In wastewater treatment plants nitrogen compounds can be removed biologically by a combination of nitrification and denitrification. There are also mechanisms to remove phosphate biologically from waste waters by introducing an anaerobic phase at the influent end of the activated sludge process. During the anaerobic phase, phosphate is released by the sludge and subsequently taken up again together with influent phosphate when oxygen is present. Phosphate release is dependent on the presence of short chain fatty acids, in particular acetate and propionate (17). Their lack will considerably diminish the rate of phosphate release. Bacteria of the genus *Acinetobacter* are in general regarded to be responsible for biological phosphate removal (8, 13, 16). It has been frequently reported that the presence of nitrate prevents an efficient phosphate release under anaerobic conditions (46). To explain the inhibiting effect of nitrate, five different hypotheses have been discussed in the literature. These hypotheses are: (i) under denitrifying conditions the pH increases and as a consequence phosphate precipitates (4, 5, 21); (ii) denitrifying and phosphate accumulating bacteria compete for growth substrates, such as fatty acids (6, 12, 17, 27); (iii) denitrifying bacteria may be capable to store large quantities of phosphate (14, 18, 29, 40, 44); (iv) nitrate as a compound inhibits phosphate release (32); and finally (v) the elevated redox potential caused by the presence of nitrate is inhibitory (35, 36). Hypothesis (i) has been tested and found not to be of relevance in the sludges we have been working with (2). This study has been initiated to test hypothesis (ii) through (v). The results are presented here.

MATERIALS AND METHODS

Sludge

Renpho sludge originated from a pilot plant built for enhanced biological phosphate removal. The pilot plant was fed with settled domestic waste water of the village of Bennekom, The Netherlands (36).

Media

The phosphate release medium contained per liter demineralized water 0.32 g NH_4Cl, 0.6 g $MgSO_4.7H_2O$, 0.07 g $CaCl_2.2H_2O$, 0.1 g EDTA, 4 g Tris.HCl, and 2 ml of a trace mineral solution (3). The pH was adjusted to 7.5 with 12 N HCl. If not otherwise stated, sodium acetate was added to a final concentration of 16 mM (1 g COD/l).

Determination of the phosphate release

The effect of nitrogen compounds on the phosphate release was determined for sludge from the end of the aerobic period. The sludge was washed once with demineralized water and resuspended in demineralized water up to a concentration of about 15 g sludge dry weight /l. The suspension was made anaerobic by vigorously gassing with a mixture of 99.5% N_2 and 0.5% CO_2. 5 ml of the anaerobic suspension was added with a syringe to sealed 39 ml serum bottles containing 20 ml 1.2 fold concentrated release medium and a headspace of 99.5% N_2 and 0.5% CO_2. After addition of the sludge, the bottles were immediately incubated in a thermostated shaker at 25°C.

Analytical methods

For dry weight determination, the biomass was centrifuged, washed once with demineralized water and dried at 100°C for 24 h. Orthophosphate (ascorbic acid method) and total phosphorus (persulphate method) were quantified according to American Public Health Association (1). Total phoshorus determinations

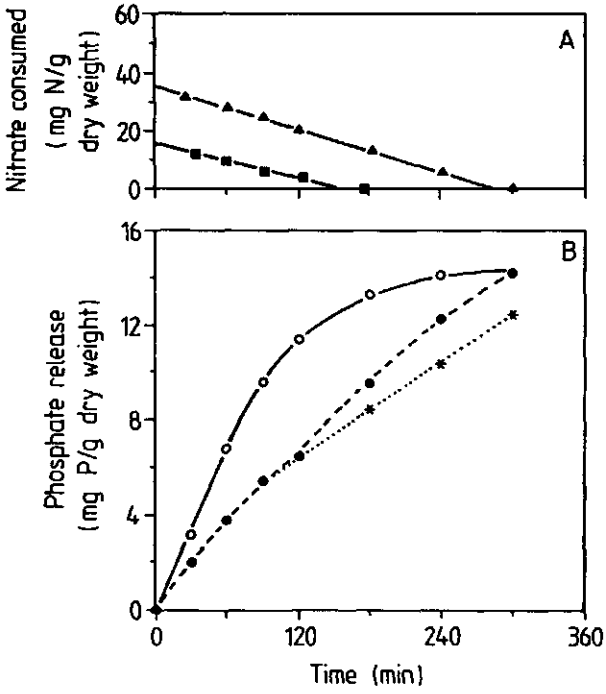

Fig. 1.(A). Nitrate consumption by Renpho sludge in the presence of 3.6 mM nitrate (■) and 7.1 mM nitrate (▲). (B). Phosphate release by this sludge without nitrate (o), with 3.6 mM nitrate (●) and with 7.1 mM nitrate (*)

were conducted in triplicate. Nitrite was measured with acidsulfonilamide (41). Nitrate was determined with alkaline salicilate (11). Acetate was measured by HPLC and quantified by differential refractometry (26). Nitrous oxide was quantified gaschromatographically with a column (110x0.21 cm) containing molecular sieve 5A (60/80 mesh), with nitrogen as carrier gas (30 ml/min), injector temperature was set at 120°C, ECD detector temperature at 300°C, oven temperature at 180°C.

Chemicals

All chemicals used were of analytical grade.

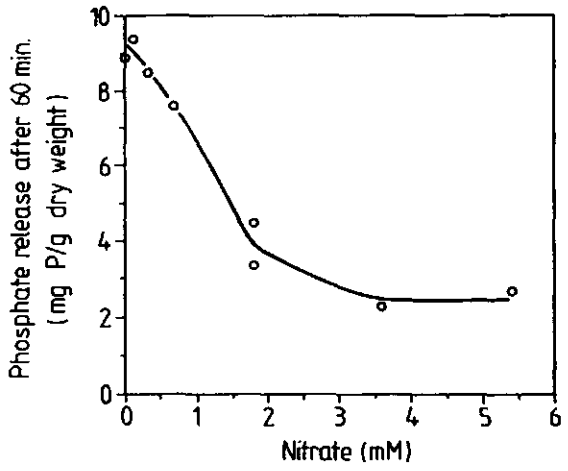

Fig. 2. Effect of increasing nitrate concentrations on the phosphate release by Renpho sludge. Samples were incubated for 60 min

RESULTS

Natural variation in phosphate release rates

In the absence of added nitrate the maximum rate of phosphate release in Renpho sludge fluctuated between 4 and 9 mg P/g dry weight.h. This fluctuation reflects variations of accumulated phosphate in sludge which is the result of changes in the domestic wastewater composition. The maximum rate within the same batch did not vary for more than 5%.

Effect of nitrate addition

Sludge samples of the Renpho plant taken at the end of the aerobic phase contained generally 8-15 mg nitrate-N/l. In the presence of added nitrate, the rate of phosphate release by washed sludge was reduced by 40 to 50% (Fig. 1). Nitrate was converted with a rate of 4 to 8 mg N/g dry weight.h, depending on the day the sludge was sampled. Nitrate was totally consumed within the period of the phosphate release experiment (Fig. 1). The phosphate release rate increased slightly after nitrate was consumed. The maximum inhibition of phosphate release by nitrate, measured after an incubation period of 60 min, was reached at nitrate concentrations of about 3.5 mM.

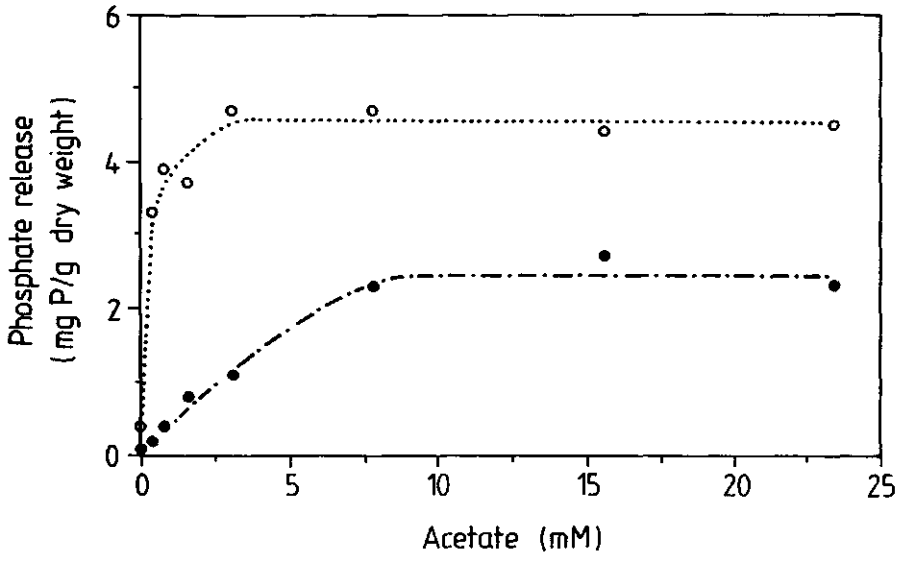

Fig. 3. Phosphate release by Renpho sludge in the presence of several acetate concentrations with (●) and without 3.6 mM nitrate (o). Samples were incubated for 60 min

Higher nitrate concentrations did not further inhibit phosphate release (Fig. 2). The production rates of nitrite and nitrous oxide never exceeded 0.2 mg N/g sludge dry weight.h during all experiments with nitrate (data not shown).

Competition for acetate

By exposing phosphate containing sludge to increasing amounts of acetate, nitrate remained inhibitory even at acetate concentrations 10 times above the theoretical amount needed for the complete denitrification (Fig. 3). Without taking microbial growth into account, 2.25 mmol acetate is necessary to reduce 3.6 mmol nitrate to nitrogen gas. In the absence of acetate almost no phosphate is released, regardless if nitrate was present or not. With Renpho sludge maximum phosphate release rates were achieved with about 3 mM acetate in the absence of nitrate and with about 8 mM in the presence of 3.6 mM nitrate (Fig. 3). No significant amounts of phosphate were chemically precipitated during the denitrification process (2).

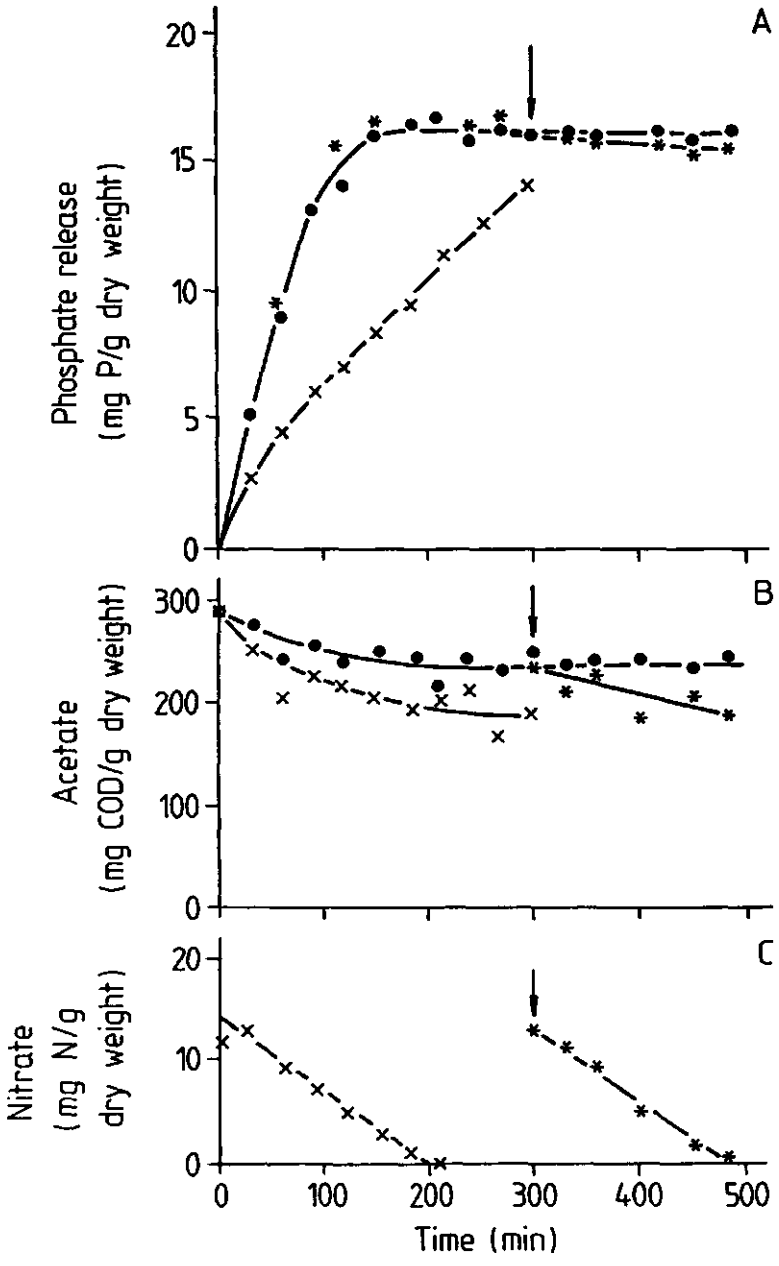

Fig. 4. Phosphate release and uptake (A), concomitant acetate consumption (B), and nitrate conversion (C) by Renpho sludge. The sludge was incubated without nitrate (♦), with 3.6 mM nitrate added at the start of the release experiment (x), and with 3.6 mM nitrate added after 300 min of phosphate release in the absence of nitrate (*). Arrow indicates nitrate addition. The results shown are averages of three independent incubations

Phosphate uptake during denitrification

In the absence of nitrate, phosphate release was completed after 3 h (Figs. 1 and 4A). Acetate was consumed with a rate of about 20 mg/g dry weight.h (Fig. 4B). If nitrate (final concentration 3.6 mM) was added 300 min after start of the release experiments, a slight phosphate uptake with a rate of about 0.2 mg P/g dry weight.h could be measured (Fig. 4A). Comparing the difference of phosphate release rates in the presence and absence of nitrate at start of the experiments (4.5 versus 9 mg P/g dry weight.h) with the rate of phosphate uptake when nitrate was added after 300 min, makes it unlikely that phosphate uptake by denitrifiers is responsible for the apparent inhibition of phosphate release by nitrate.

The uptake of phosphate after nitrate addition at time 300 min can be explained by growth of denitrifiers. Theoretically, 90 mmol nitrate (25 ml of a 3.6 mM solution) would allow the growth of about 3 mg biomass which would consume 42 mg P. In the experiments depicted in Fig. 4A about 45 mg P is consumed (0.6 mg P/g dry weight translates to 45 mg P/75 mg dry weight; each bottle contained 75 mg sludge dry weight only). The theoretical values were calculated based on the following assumptions: 14 mg P is needed for the synthesis of one gram biomass (3) and 32.5 g biomass is formed per mol nitrate denitrified (42). As a consequence of these calculations, growth of denitrifiers cannot cause the observed inhibition of phosphate release by nitrate in our system.

Acetate and nitrate were converted with rates of about 20 mg/g dry weight.h and 4 mg N/g dry weight.h, respectively. Interestingly, a slightly higher acetate consumption rate was observed (30 mg/g day weight.h) when nitrate was added after 300 min but the nitrate consumption rate remained about constant (Figs. 4B and C).

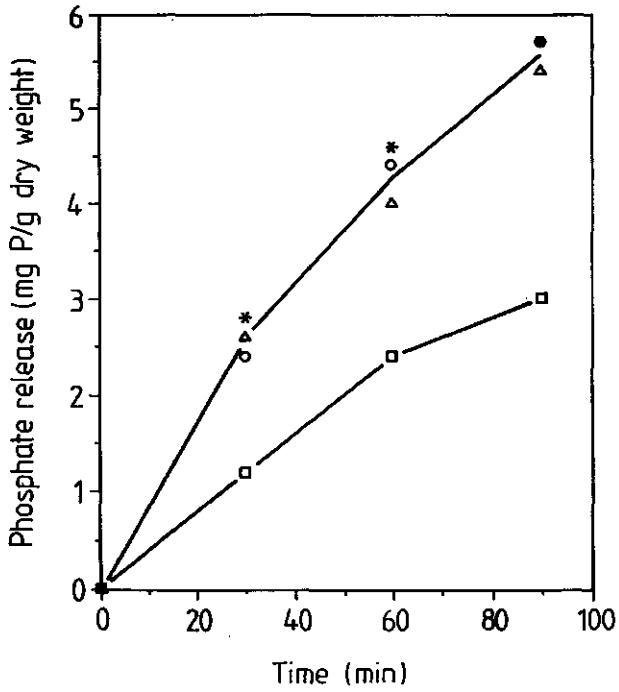

Fig. 5. Initial phase of phosphate release by Renpho sludge in the presence of nitrate (3.6 mM) plus 5 mM KCN. No nitrate or KCN added (△), with nitrate (□), with KCN (○), with KCN plus nitrate (*). Without KCN, nitrate was depleted after 120 min, with KCN nitrate was not consumed

Direct effect of nitrate on phosphate release

In the presence of KCN (5 mM) phosphate release from Renpho sludge was not affected by the presence of nitrate (3.6 mM) (Fig. 5). KCN completely inhibited denitrification (not shown). The same results were obtained with sodium azide (2.5 mM) another inhibitor of denitrification (not shown).

The effect of different nitrogen oxides and hydroxylamine on phosphate release

Nitrite, nitric oxide and nitrous oxide are intermediates of the denitrification. Nitric oxide production has also been reported to occur upon anaerobic incubation of nitrifying bacteria with nitrite (37). Hydroxylamine (NH_2OH) is an

Table 1. Phosphate release by Renpho sludge, determined after 60 min in the presence of several nitrogen oxides and hydroxylamine. The data given are averages of two independent incubations. Deviations from the means are also included

Additions	Concentration (mM)	Phosphate release (mg P/g dry weight)
None (control)		7.7 ± 0.0
Nitrate	3.6	4.8 ± 0.8
Nitrite	3.6	4.1 ± 0.2
Nitric oxide	0.3[1)]	3.4 ± 1.3
Nitrous oxide	3.6[1)]	7.7 ± 0.1
NH_2OH	3.6	6.5 ± 0.2

[1)]Concentration in the liquid phase

intermediate of assimilatory reduction of nitrate (15). The nitrogen oxides and hydroxylamine were tested for their potential to inhibit phosphate release in Renpho sludge (Table 1). Nitric oxide at 0.3 mM already inhibited phosphate release by 40%. Nitrite showed about the same effect as nitrate. Hydroxylamine only slightly reduced phosphate release, whereas nitrous oxide did not show any effect. The inhibitory effect of nitric oxide on the phosphate release was slightly reduced if 5 mM KCN was added along with or 30 min after the addition of 0.3 mM nitric oxide (Fig. 6). Nitric oxide was also inhibitory when sludge was exposed to it after a 30 min preincubation with 5 mM KCN. Nitric oxide did not react with KCN since its concentration remained constant in control incubations with 5 mM KCN but without sludge.

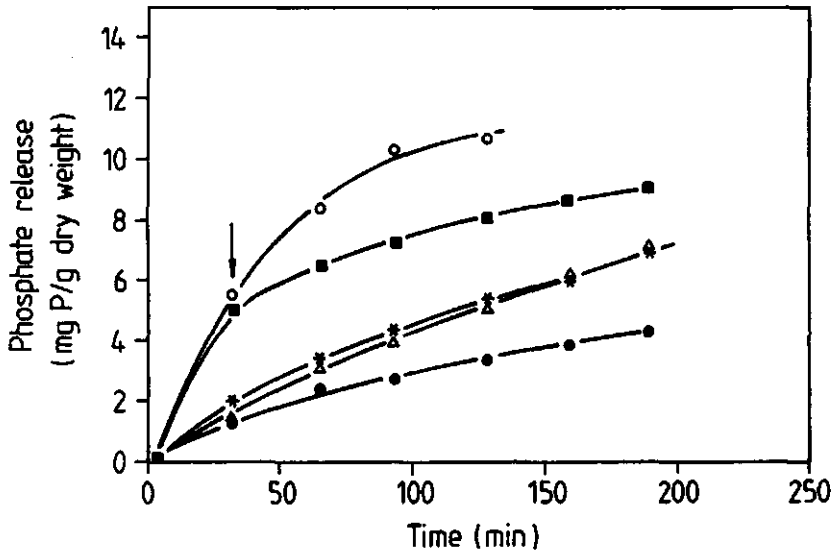

Fig. 6. Phosphate release by Renpho sludge in the presence of 0.3 mM nitric oxide (●), 0.3 mM nitric oxide plus 5 mM KCN (*), 0.3 mM nitric oxide and addition of KCN at t = 30 min (▲); 5 mM KCN and addition of nitric oxide at t = 30 min (■), no additions (○). Arrow indicates addition of nitric oxide or KCN

DISCUSSION

Introduction, optimization and process control of biological phosphate removal is still largely based on hypotheses and empirical data. One of the major problems for an efficient biological removal of phosphate is the adverse effect of nitrate.

In the presence of nitrate, the phosphate removal capacity of sludge decreases with time (3, 21). A number of hypotheses have been put forward to explain the negative influence of nitrate. However, none of these hypotheses could satisfactorily explain the nitrate effect in the sludge used in this investigation.

Some data presented here are at variance with published studies. Iwema and Meunier (27) and Mostert et al. (34) found that the nitrate effect could drastically be reduced by the

addition of excess acetate. The first group worked with activated sludge from a laboratory plant fed with a synthetic but ill-defined medium. The medium contained among others acetate. The sludges, used by the second group, originated from wastewater treatment plants with Bardenpho process configurations. The reason for the observed differences is not clear. Schön and Streichan (40) suggest that the quantitative disagreements between various authors on the effect of nitrate on phosphate release, is due to differences in composition of the microbial populations in the sludges investigated. According to these authors, the presence or absence of nitrification in plants from which the sludge was sampled is of importance for the nitrate effect.
Lötter et al. (33) isolated phosphate accumulating bacteria able to denitrify. The same group of scientists reported earlier that about 50% of *Acinetobacter* isolates convert nitrate into nitrite (31). However, other groups failed to isolate phosphate accumulating bacteria using nitrate as terminal acceptor (13, 22). Recently, Kuba et al. (30) clearly showed biological phosphate removal under denitrifying conditions when the entire process was run anaerobically and nitrate replaced oxygen as terminal electron acceptor.

The formation of free nitric oxide has long been doubted but with the development of new techniques and more sensitive detection methods, nitric oxide formation has been found in a variety of denitrifying bacteria (9, 19, 20, 38, 47). It is now accepted that nitric oxide is a free intermediate of denitrification (24, 50). Our knowledge about its formation in natural populations is very limited. For pure cultures of denitrifying bacteria, evidence has been presented that the conversion of nitrite to nitrous oxide is a two step process, catalyzed by two separate enzymes with nitric oxide as free intermediate (9, 10, 23, 25, 49). Nitric oxide production by bacteria of the *Nitrosomonas* group could also be measured under both, oxic and anoxic conditions (25, 37, 50). Nitric oxide formed in acidic as well as slightly alkaline soils did

not occur after sterilization which indicated that biological processes were responsible for its formation (39). In a next paper, we present evidence for nitric oxide production by activated sludge.

Nitric oxide can also be formed by chemodenitrification (7, 43). Here, nitrite is reduced to nitric oxide spontaneously with electrons from ferrous iron or other reduced metal ions (7, 45). The presence of these ions in sludge might at least partially be responsible for nitric oxide formation. Therefore, high iron concentrations in sludge in combination with nitrate or nitrite could be detrimental for biological phosphate removal.

Cyanide and azide prevented the inhibition of phosphate release by nitrate. Both compounds inhibit nitrate reductases (24, 28) and as a result reduction of nitrate to nitric oxide. The experiment with cyanide and azide clearly showed that inhibition of phosphate release by nitrate is not the result of the presence of the nitrate ion or its action as redox partner in the chemistry of the medium.

The mode of action of nitric oxide in phosphate release is not known. However, some indications of the mechanism can be deduced from the data shown in Fig. 6. Cyanide slightly reduces the nitric oxide inhibition, regardless whether cyanide or nitric oxide was first added. Both cyanide and nitric oxide form complexes with iron (7, 48) and as a consequence with iron containing enzymes. It might well be that cyanide displaces some of the nitric oxide from its place of action, the cyanide complex being less inhibitory than the nitric oxide complex.

In conclusion we can say that the negative effect of nitrate on biological phosphate removal in sludge investigated here, is the result of its conversion to nitric oxide. More research is required on the mode of action of nitric oxide in the process of phosphate release and on the factors affecting the formation of nitric oxide. This information is needed for an

optimal implementation of the biological phosphate removal into the wastewater treatment system.

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Chapter 5

Nitric oxide production by sludge removing biologically phosphate and its effect on the anaerobic phosphate release

NITRIC OXIDE PRODUCTION BY SLUDGE REMOVING BIOLOGICALLY PHOSPHATE AND ITS EFFECT ON THE ANAEROBIC PHOSPHATE RELEASE

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ABSTRACT

Nitric oxide production by sludges removing biologically phosphate was dependent on the sludge type, the nitric oxide precursor, the carbon source and the pH. At pH 7.5, rates of nitric oxide production by sludge from a pilot plant treating municipal waste water were 390 μmol/g sludge dry weight.h upon incubation with acetate plus nitrite, 16 μmol/g dry weight.h with acetate plus nitrate and 25 μmol/g dry weight.h with glucose plus nitrate. The highest rate of nitric oxide production upon incubation with acetate plus nitrite was 550 μmol/g dry weight.h at pH 8. At pH 7.5, rates of nitric oxide production by sludge originated from lab-scale installations fed with synthetic media were 100-160 μmol/g dry weight.h upon incubation with acetate plus nitrite, 6-7 μmol/g dry weight.h, with acetate plus nitrate and 13-16 μmol/g dry weight.h with glucose plus nitrate. Pasteurized sludge produced nitric oxide only upon incubation with nitrite (12 μmol/g dry weight.h), but not with nitrate. Phosphate release by sludge removing biologically phosphate was reduced by 50% at a nitric oxide concentration of 30 μM. The results presented in this paper show that nitric oxide is formed by biological processes in sludges from wastewater treatment plants removing biologically phosphate and that in the systems tested nitric oxide is responsible for the reduced phosphate release under denitrifying conditions.

INTRODUCTION

Activated sludge can be enriched with phosphate accumulating bacteria by introducing an anaerobic zone at the front end of an activated sludge plant. During the anaerobic phase phosphate is released by the sludge and subsequently taken up again together with influent phosphate when oxygen is present. This can result in effluent concentrations as low as 0.1 mg P/l (41). In general, bacteria of the genus *Acinetobacter* are considered to be responsible for the phosphate removal (e.g. 8, 14, 16). Phosphate and nitrogen compounds are frequently removed simultaneously during wastewater treatment. The removal of nitrogen compounds can be achieved by a succession of nitrification and denitrification. The presence of nitrate during anaerobiosis has been reported to diminish the success of the biological phosphate removal. Nitrate inhibits the phosphate release. An efficient phosphate release is necessary to maintain enhanced phosphate removal in wastewater treatment plants (21). In the sludges we have investigated, it was not nitrate but in fact its reduction product nitric oxide that reduces the release of phosphate (3). The present study was undertaken to determine whether nitric oxide is actually produced during denitrification in sludges removing biologically phosphate and whether its steady state concentration is high enough to inhibit phosphate release in these sludges.

MATERIALS AND METHODS

Biomass

Renpho sludge originated from a pilot plant with enhanced biological phosphate removal fed with settled domestic waste water of the village of Bennekom, The Netherlands (30). Fill and draw system sludges were grown on a synthetic medium in the presence or absence of nitrate (2).

Media

The phosphate release medium (pH 7.5) contained per litre demineralized water 0.32 g NH_4Cl, 0.6 g $MgSO_4.7H_2O$, 0.07 g $CaCl_2.2H_2O$, 0.1 g EDTA, 4 g Tris, 2.15 g/l sodium acetate.$3H_2O$, and 2 ml of a trace metal solution (2). Nitric oxide production was determined in release medium to which 4.3 g/l sodium acetate.$3H_2O$ or 2 g/l glucose.H_2O were added. pH values were adjusted with 1 N HCl. pH did not change during the course of the experiments.

Experimental set-up

The experiments were performed with sludges from the end of the aerobic period. The sludges were washed once with demineralized water and resuspended in demineralized water to make a final biomass concentration of about 15 g dry weight/l.

Batch experiments with variable nitric oxide partial pressures: Bottles containing the sludges were made anaerobic by flushing 15 min with argon. They were sealed and subsequently stored on ice before use. 5 ml of the sludges suspension was added to 39 ml serum vials, sealed with butyl rubber stoppers, containing 20 ml 1.2 fold concentrated release medium. Before sludge addition, these serum vials were also made anaerobic with argon. Nitric oxide was added up to the desired concentration with gas tight syringes (Schinkel Meetinstrumenten B.V., Nieuwegein, The Netherlands). After addition of nitric oxide and the sludge, the vials were immediately incubated in a thermostated shaker at 25°C.

Batch experiments at constant nitric oxide partial pressures: Gas mixtures containing nitric oxide were bubbled at a flow rate of 4 to 12 l/min through 50 ml sludge in release medium with acetate placed in a vessel with a side arm (Fig. 1). The original gas mixture contained 3.8% nitric oxide and 96.2% nitrogen. Depending on the partial pressure of nitric oxide desired, the original gas mixture was diluted with nitrogen gas. For an efficient gas transfer, the sludge was stirred and

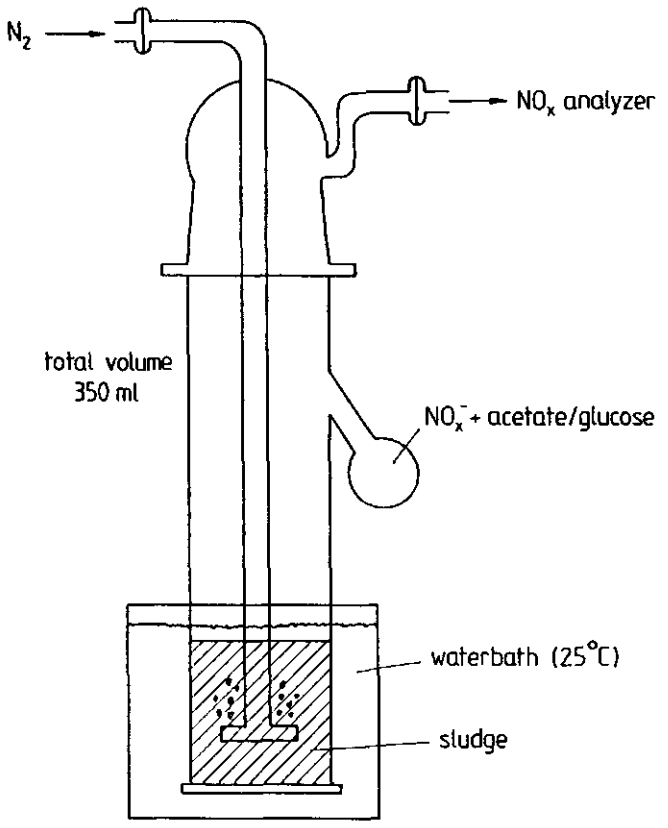

Fig. 1. Reaction vessel for continuous measurement of nitric oxide production

the gas mixture was introduced into the liquid phase by a sintle stone. The pretreatment of the biomass was the same as described above, with the exception that a mixture of 0.5% CO_2 and 99.5% N_2 were used instead of argon to make the sludge anaerobic. Prior to the addition of 10 ml sludge suspension to 40 ml 1.2 fold concentrated release medium, the vessel was flushed for 5 min with the desired nitric oxide/nitrogen mixtures.

Continuous analysis of nitric oxide production: The sludge was concentrated as descibed above, flushed with the N_2/CO_2 (99.5/0.5) mixture and stored on ice before use. 5 ml of this sludge was added to 18 ml of 1.4 fold concentrated release medium in a vessel with side arm (Fig. 1). The sludge

suspensions were stirred and flushed with nitrogen gas. After gassing with nitrogen for 5 min, 2 ml of a solution containing nitrate or nitrite together with glucose or acetate was added from the side arm. Nitric oxide was continuously monitored in the nitrogen gas stream. For the determination of abiotic nitric oxide production, sludge was replaced by oxygen-free solutions of iron (II) or iron (III) chloride, final concentration 1 mM.

Analytical methods

For dry weight determination the biomass was centrifuged, washed once with demineralized water and dried at 100°C for 24 h. Orthophosphate (ascorbic acid method) and total phosphorus (persulphate method) were quantified according to American Public Health Association (1). Total phoshorus determinations were conducted in triplicate. Nitrite was measured with acid sulfonilamide (36). Nitrate was determined with the alkaline salicilate method (12). Nitrous oxide and nitric oxide were quantified gaschromatographically with a Chrompack 438A (Chrompack B.V., Middelburg, The Netherlands) with an electron capture detector connected to a molecular sieve 5A (60-80 mesh) column (110x0.21 cm). Nitrogen was the carrier gas (30 ml/min), injector temperature was set at 120°C, detector at 300°C, oven at 180°C. The concentration of the nitrogen oxides was calculated using the Bunsen absorption coefficients at 25°C of 0.045 ml/ml for nitric oxide and of 0.45 ml/ml for nitrous oxide (28). Nitric oxide was also continuously measured with a Nitrogen Oxide Analyzer (model 8840 ML, Monitor ABS INC., Santiago-California, USA). In this method the detection of NO is based on the chemoluminescence of excited NO_2 which is produced during the reaction of NO with O_3. Total iron in sludge was quantified after boiling 0.2 ml sludge with 0.4 g sodium peroxodisulphate in 5 ml 0.6 N H_2SO_4. After cooling, 2.8 ml 2 N NaOH, demineralized water and 5 drops of Ferrospectral II (E. Merck Nederland B.V., Amsterdam, The Netherlands) were added to make a total volume of 10 ml. The extinctions were measured spectrophotometrically at 565 nm

and were corrected for the extinctions of boiled solutions without sludge. The population density of denitrifying bacteria in sludge was determined by most probable number (MPN) analysis in a medium with 42 mM acetate supplemented with 4 g KNO_3/l (40). Before dilution, the sludge was sonified for 30 s at 30 W (Model B12, Branson Sonic Power Compagny, Danbury-Conn., USA). During sonification the sludge was kept on ice.

Chemicals

All chemicals used were of analytical grade. The gasses were of industrial quality (Hoek Loos B.V., Schiedam, The Netherlands).

RESULTS

Sludges

Different sludges were used, because the different behaviour of the anaerobic phosphate release by the sludges towards the presence of nitrate. With 5 mM glucose plus 3.5 mM nitrate, phosphate release by all sludges was entirely inhibited (Table 1). With 16 mM acetate plus 3.5 mM nitrate, the inhibition was dependent on the sludge type used (Table 1). Without nitrate, the phosphate release with 5 mM glucose was at about half the rate of the release with 16 mM acetate (results not shown).

Nitric oxide consumption by Renpho sludge

Nitric oxide disappeared immediately after addition at a rate of 0.6-1.0 mmol/g sludge dry weight.h (Fig. 2). The disappearance of nitric oxide was accompanied by a non-stoichiometrical accumulation of small amounts of nitrous oxide (29-46 μmol/g sludge dry weight.h). No attempts were made to quantify the production of nitrogen gas.

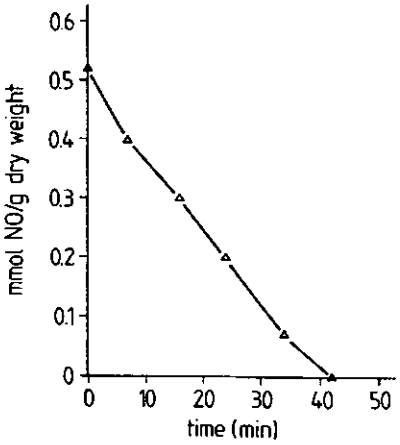

Fig. 2. Nitric oxide consumption by sludge from the Renpho pilot plant after addition of 0.5 mmol nitric oxide/g dry weight

Nitric oxide production by activated sludge

No nitric oxide was measured gaschromatographically in batch experiments with sludge from the Renpho plant incubated in releasemedium containing 7 mM nitrate, though phosphate release was inhibited by 40 to 50% in presence of added nitrate. With a sludge concentration of 5 g dry weight/l still no nitric oxide could be seen with this method. The gaschromatic method used in this study had a detection limit of 0.1% nitric oxide.

When stripping Renpho or fill and draw sludges containing nitrate or nitrite with dinitrogen gas, nitric oxide production was always observed. The nitric oxide production rates increased with time (Fig. 3). A maximum production rate (V_{max}) with nitrate was obtained after about 15 min (Fig. 3), with nitrite after 2-3 min (not shown). The V_{max} values were measured at a flow rate of 1 l/min nitrogen-gas. The apparent V_{max} were about 70% of the effective V_{max} values, which could be obtained at flow rates of more than 3 l/min nitogen gas only. This is in accordance with the observations of Remde *et al.* (33), when stripping nitric oxide from soil samples.

The apparent V_{max} values depended on the type of nitrogen oxide, the carbon compound and the sludge used (Table 1). Renpho sludge produced nitric oxide with a rate of 390 µmol/g

Table 1. Apparent maximum nitric oxide production rates (V_{max}) expressed as μmol NO/g sludge dry weigt.h during incubation with nitrate or nitrite (7 mM). The inhibition of the phosphate release upon incubation with nitrate or nitrite are also shown. The data shown are the mean of two independent experiments

Sludge	P-content (%P)[1]	V_{max}	Time[2] (min)	Inhibition of phosphate release (%)
Incubated with acetate and nitrate[3]				
Renpho	3	16	15	47
Fill and draw system (standard medium)	7	6	12	13
Fill and draw system (grown in the presence of NO_3^-)	5	7	20	29
Incubated with acetate and nitrite[3]				
Renpho	3	390	2	47
Fill and draw system (standard medium)	7	100	3	13
Fill and draw system (grown in the presence of NO_3^-)	5	160	2	29
Incubated with glucose and nitrate[4]				
Renpho	3	25	15	100
Fill and draw system (standard medium)	7	16	12	100
Fill and draw system (grown in the presence of NO_3^-)	5	13	20	100

[1]Based on sludge dry weight

[2]Time elapsed to obtain V_{max}

[3]Phosphate release inhibition determined with acetate

[4]Phosphate release inhibition determined with glucose

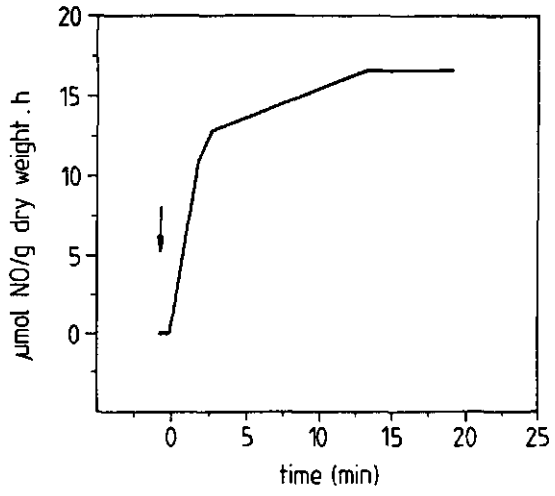

Fig. 3. Increase of the nitric oxide production velocity during incubation of sludge from the Renpho pilot plant with 7 mM nitrate. The arrow indicates the point of nitrate and acetate addition

dry weight.h upon incubation with nitrite plus acetate, 25 µmol/g dry weight.h with nitrate plus glucose and 16 µmol/g dry weight.h with nitrate plus acetate. The apparent V_{max} values of the fill and draw system sludges were 1.6-3.9 fold lower than these measured with Renpho sludge.

Abiotic nitric oxide production

Nitric oxide can be formed from nitrate by a combination of biologically catalyzed and spontaneous reactions (7). In these reaction sequences, microorganisms convert nitrate into nitrite and iron(III) into iron(II). Nitrite and iron (II) can react spontaneously to iron (III) and nitric oxide in a process called chemodenitrification (7). Nitric oxide can further be reduced by iron (II) to nitrous oxide. Renpho sludge contained about 0.1 mmol iron/g dry weight. This concentration was about ten times higher than the concentration in the fill and draw sludges, which might explain the lower V_{max} values of these sludges as compared to the Renpho sludge. Pasteurized Renpho sludge produced nitric

Table 2. Abiotic nitric oxide production rates (V_{max}) with 7 mM NO_x at pH 7.5. The data shown are the mean of two independent experiments

Addition to release medium			V_{max} (Mmol/l.h)	Time[1] (min)
Sludge[2]	+	$NaNO_3$	0	
Sludge[2]	+	$NaNO_2$	13.7	8
$FeCl_3$ (1 mM)	+	$NaNO_3$	0	
$FeCl_3$ (1 mM)	+	$NaNO_2$	27.1	2
$FeCl_2$ (1 mM)	+	$NaNO_3$	0	
$FeCl_2$ (1 mM)	+	$NaNO_2$	25	9

[1]Time elapsed to obtain V_{max}

[2]Pasteurized Renpho sludge (1.2 g dry weight/l)

oxide during incubation with nitrite but not with nitrate (Table 2). The rate of this nitric oxide production was 28 times slower as compared to the living sludge (Table 1).

Abiotic nitric oxide production occurred during incubation of iron (II) or iron (III) with nitrite, but not with nitrate (Table 2). Nitric oxide produced with iron (III) may be a result of the following reaction sequence: Iron (III) and nitrite are converted to iron (II) and nitrate, a reaction which is thermodynamically possible at pH 7 ($\Delta G^{0\prime} \approx -105$ kJ/reaction). The produced iron (II) reacts with nitrite to form nitric oxide and iron (III). No attempts were made to quantify nitrate.

Abiotic and biological nitric oxide formation were both pH dependent (Fig. 4). At pH values between 7.0 and 8.5, the sludge produced nitric oxide at maximal rates. This corresponds with optimum pH values reported for denitrification (27). The increase of the nitric oxide production at a pH below 7.0 might be due to statistical

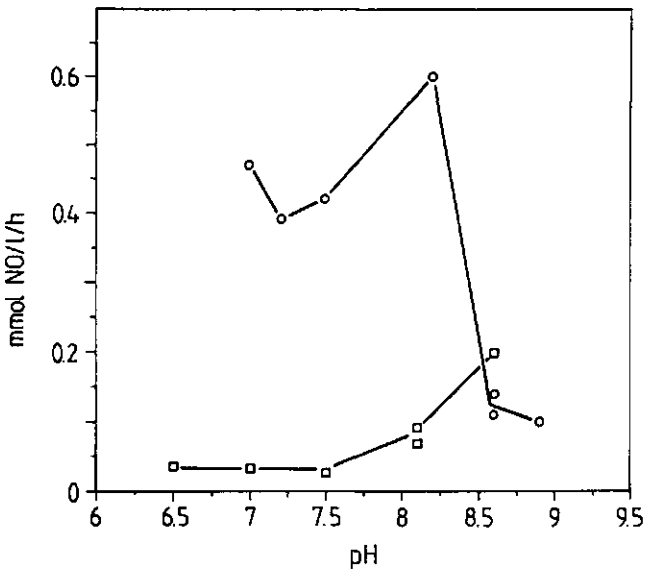

Fig. 4. Dependency of the Vm value on pH. (O): The Vm values determined with Renpho sludge (1.1 g/l) upon incubation in release medium with acetate plus 7 mM nitrite; (□): The Vm value determined for the chemical reaction between 1 mM $FeCl_2$ plus 7 mM nitrite. Results are from single measurements

variations in the measurements. At pH values higher than 7.5 the rate of abiotic nitric oxide formation increased.

Effect of nitric oxide on the phosphate release

Nitric oxide reduced the phosphate release by 50% at a concentrations of 140 μM in the liquid phase when nitric oxide was added in a batch experiment or already at 30 μM when nitric oxide concentration was kept constant by continuous flushing (Fig. 5). The higher concentration at which nitric oxide became inhibitory for the phosphate release in batch experiments can be explained by consumption of nitric oxide (Fig. 2).

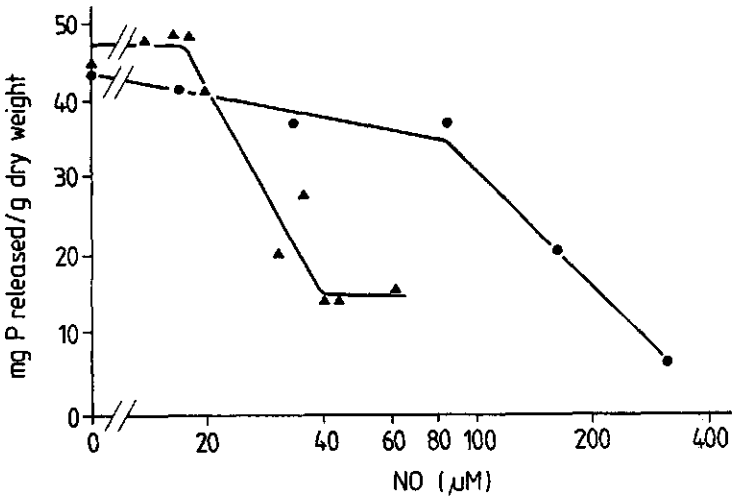

Fig. 5. Inhibition of phosphate release in fill and draw system sludge by nitric oxide. Nitric oxide concentrations initially present (●); Nitric oxide concentrations were kept constant by continuously gassing the incubation mixtures (▲). Phosphate release was determined after an incubation period of 60 min

DISCUSSION

Nitric oxide formation by activated sludges was observed during incubations with nitrate and nitrite. This is, as far as we know, the first time that nitric oxide production has been detected in these ecosystems. The reduction of nitric oxide and its formation under denitrifying conditions are the least well characterized steps associated with denitrification (23). It has been proposed to exclude nitric oxide as intermediate of the denitrification (4). To explain a number of observations, two distinct pathways for denitrification have been proposed, one with and one without nitric oxide as intermediate (17, 23). However, increasing evidence made it clear that nitric oxide should be regarded as an intermediate of denitrification. These evidences were:
(i) transposon mutants defective in nitrite reductase showed the same nitric oxide reductase activity as their parental strains (43) and (ii) studies with purified or partial purified enzymes showed that nitric oxide is a free

intermediate in denitrification (11, 22, 24, 44). The failure to detect nitric oxide as intermediate in earlier studies has been ascribed to the high affinity of nitric oxide reductase for its substrate (10, 19, 32), to its inhibition of its own formation (15, 34) and to the presence of compounds in assay mixtures reducing nitric oxide to nitrous oxide (23, 44). Production of nitric oxide by several bacterial cultures has been shown by stripping cultures with nitrogen gas just as described here and by spectrophotometric methods, which are based on the trapping of nitric oxide with extracellularly added hemoproteins (11, 18, 19, 32, 42).

Comparison of published nitric oxide production rates with our own results show Renpho sludge to have the highest rates (Table 3).

This high rate might be due to the difficulty to fully dispers sludge flocs into single cells, consequently underestimating the number of denitrifying bacteria.

Besides purely enzymatically, nitric oxide can also be formed by a combination of enzymatic nitrite formation and chemodenitrification (7, 39). The results with pasteurized sludge showed that the rate of spontaneously nitric oxide

Table 3. Nitric oxide production rates by nitrifyers, denitrifyers and in two habitats

Habitat/organism	Nitric oxide production ($fmol.h^{-1}.cell^{-1}$)	Reference
Nitrifying bacteria	16-53	(31)
Denitrifying bacteria	1.3-355	(32)
Soils[1)]	70	(32)
Renpho sludge[2)]	200-2000	this paper

[1)]Results were obtained with a alkaline and a acidic soil, which had about the same production rates. The population size was estimated by most probable number.

[2)]The number of denitrifying bacteria was estimated by most probable number as described in Materials and Methods

formation from nitrite is small in our system when compared to the rates found in living sludge (Table 1 & 2). One of the most significant reactions of chemodenitrification is the acid-catalysed disproportioning of nitrite into nitric oxide and nitrate (38):

$$3NO_2^- + 2H^+ \rightarrow NO_3^- + 2\,NO + H_2O \qquad (1)$$

This reaction is stimulated by the presence of ferrous iron but is thought not to be possible to proceed spontaneously above pH 5.47 (39). In acidic media ferrous ascorbate catalyses the chemical reduction of nitrite to nitric oxide, a reaction virtually non existent at pH > 6 (44). In neutral and alkaline solutions nitrous oxide was the major product of the reaction between ferrous ascorbate and nitrite, reaching a maximum at pH 7. Both nitric oxide and nitrous oxide were found to be produced between pH 4 and 9, with maximum production rates below pH 5 (6). In a reaction mixture containing nitrite, Fe (III) oxyhydroxide (FeOOH) and ferrous iron, nitrous oxide formation started at pH 7.5 and the rate increased with increasing pH values (35). With only ferrous iron and nitrite, nitrous oxide formation also occurred, but at a slower rate. In distilled water with ferrous sulfate and nitrite, nitrous oxide plus dinitrogen were found as gaseous products at pH 8, whereas at pH 6 nitrous oxide plus trace amounts of nitric oxide were detected (29).

In this study the formation of nitric oxide by the ferro-iron dependent chemodenitrification was found to increase at pH values above 7.5 (Fig. 4). Thermodynamically, formation of nitric oxide is possible at these pH values, when ferrous iron is oxidized to FeOOH or $Fe(OH)_3$ (Table 4). The free energy values of these reactions become more negative at increasing pH values. The reactions with Fe^{3+} or Fe_2O_3 (magnetite) as products are endothermic and will thus not proceed spontaneously (Table 4). Nitric oxide can further react with ferrous iron to form nitrous oxide and FeOOH, $Fe(OH)_3$ or Fe_2O_3. The free energy values of these reactions become more exothermic with increasing pH values, and are therefore base catalysed. Ferrous iron and nitric oxide can form a [Fe^{2+}-NO]

complex (7). The binding constant between ferrous iron and nitric oxide in this complex seems to be relatively low, since bacteria can still oxidize ferrous iron, thereby releasing nitric oxide. In the chemodenitrification systems, where at high pH values no nitric oxide formation was detected, the complex and the formation of nitrous oxide by the reaction between ferrous iron and nitric oxide might have caused the failure to find nitric oxide formation. In stripping systems, liberation of nitric oxide from the complex seems to be possible due to the high gas sparging rates, preventing its further conversion into nitrous oxide.

Nitric oxide has been shown to be toxic for many microorganisms at concentrations higher than 0.2 mM in the liquid phase (5). In mammalia, it causes relaxation of smooth muscles via a heme dependent activation of cytosolic guanylate cyclase, catalyzing the conversion of GTP in cyclic-GMP (25, 26). Nitric oxide inhibited the lactate driven nitrate respiration by an *Escherichia coli* strain, possibly due to reactions with non-heme iron (7). Nitric oxide inhibited the nitrous oxide respiration of several denitrifying bacteria via an unknown mechanism (23). It inhibits the oxidase activity and nitrite reductase (9, 12), succinate dehydrogenase (9) and apparently phosphate release by enhanced biological phosphate removal sludge, though the precise inhibition mechanism is not known. At this stage even a

Table 4. Calculated free energies (kJ mol^{-1}) for the redox reactions between nitrite and ferrous iron at pH 8

Reaction	ΔG (pH=8)[+)]
$Fe^{2+} + NO_2^- + 2H^+ \rightarrow Fe^{3+} + NO + H_2O$	+52
$3Fe^{2+} + 3NO_2^- + H_2O \rightarrow Fe_3O_4 + 3NO + 3H^+$	+42
$Fe^{2+} + NO_2^- + H_2O \rightarrow FeOOH(amorph) + NO + H^+$	-58
$Fe^{2+} + NO_2^- + 2H_2O \rightarrow Fe(OH)_3 + NO + H^+$	-58

[+)]The free energy of formation were obtained from Stumm and Morgan (37), except for nitric oxide, which is from Chang (13).

hypothesis for a likely mechanism can not be given.

The results presented in this study show that nitric oxide is formed in sludges under denitrifying conditions. Its formation can explain the inhibition of phosphate release in the presence of nitrate. For a successful implementation of the biological phosphate removal process in the wastewater treatment systems more knowledge is needed on the mode of action of nitric oxide on the phosphate release system. Moreover, nitric oxide is a potent greenhouse and air pollution gas destructing ozone (20). Therefore its production in wastewater treatment plants should obtain more attention.

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Chapter 6

Concluding remarks

CONCLUDING REMARKS

The results described in this thesis may leave some questions about the applicability of the results for wastewater treatment plants. Hence, an attempt will be made in this chapter to discuss the results more in relation with its possible use for engineers working with pilot- and full scale plants.

The concept of the fill and draw system, as described in chapter 2, can be easily adopted for optimizing biological phosphate removal in real wastewater treatment plants. The system can be placed nearby a full scale wastewater treatment plant, which has to be adapted for biological phosphate removal. With sewage as influent for the fill and draw system, parameters as length of the anaerobic phase and aerobic phase can be studied without the risk of deterioration of the effluent of the full scale treatment plant. Because the relatively low financial investments which are necessary for the fill and draw systems, more than one of them can be used in parallel for optimization of the process. In this way, several parameters can be varied simultaneously. The different results can be better compared, because conditions, as e.g. variations in the sewage composition, fluctuate to the same extent.

Many engineers are developing simulation models for designing wastewater treatment plants. Such simulation models can give predictions for the nutrient removal under a variety of conditions. Most simulation models are based on input and output data of nutrients in wastewater treatment plants and take bacterial growth parameters into account. This thesis contains several data, which can be used to adapt existing models to enhanced biological phosphate removal:

- Enrichment cultures contained phosphorus up to 110 mg P/g dry weight (chapter 1). This high phosphorus content in combination with phosphate release can be used as indication for the maximum amount of phosphorus accumulated by sludges

almost completely composed of polyphosphate accumulating bacteria. This parameter might be used for estimating of the number of polyphosphate accumulating bacteria necessary for obtaining the desired phosphorus concentrations in effluents of wastewater treatment plants.

- Sludges devoid of spontaneously precipitated phosphate contained about 15 mg P/g dry weight after the release of phosphorus (Chapter 2).
- Treatment of sludges with perchloric acid was very appropriate for estimating the amount of phosphates spontaneously precipitated on sludges (chapter 3).

The stimulating effect of acetate on the phosphate release and on the development of a phosphate accumulating bacterial community in sludge confirmed earlier research that acetate is important for obtaining biological phosphate removal in wastewater treatment plants (chapter 1 & 2). However, the biochemical relation between acetate uptake, polyhydroxybutyrate accumulation and phosphate release during anaerobic conditions could not satisfactorily be elucidated (chapter 2). To understand this complex mechanism, studies with pure bacterial cultures are necessary. Although the pure cultures of *Acinebacter* used in our investigation could accumulate high amounts of phosphate, the properties of these bacteria were significantly different from those of the sludges we have worked with (chapter 3). Attempts to isolate bacteria from the enrichment cultures with the same properties as biological phosphate removing sludges failed (not shown). Sonified sludges were plated on agar medium with acetate as carbon source. Isolates were selected microscopically and with staining on polyphosphate accumulation. All cultures lost the polyphosphate accumulating property after one or two transfers on agar plates (purity checks).

To prevent eutrofication in fresh, estuarine and marine waters, removal of both phosphate and total nitrogen (ammonia and nitrate) are required in wastewater treatment plants. However, the presence of nitrate in the anaerobic zone of a

wastewater treatment plant is seen as one of the major problems for obtaining biological phosphate removal in wastewater treatment plants with alternating oxic and anoxic conditions. The negative effect of nitrate on the biological phosphate process was confirmed by the lower amount of phosphate accumulated by sludge from fill and draw systems fed with synthetic medium supplemented with nitrate (chapter 2) and by the inhibition of phosphate release by Renpho sludge in the presence of acetate plus nitrate (chapter 4). Nitric oxide was found to be the cause of the negative effect of nitrate on the phosphate release (chapter 4 & 5). The formation of this inhibitor implicates that enhanced biological phosphate removal and total nitrogen removal can only take place in wastewater treatment plants where the anaerobic period is kept sufficiently long allowing both nitric oxide conversion into other nitrogen compounds and phosphate release. With longer anaerobic periods sufficient substrates in sewage are needed to sustain both phosphate release and denitrification.

The discovery that nitric oxide and nitrous oxide are both produced in the activated sludge process shows that wastewater treatment plants can participate in the emission of these nitrogen oxides into the atmosphere. Gaseous nitrogen oxides are greenhhouse gasses and co-responsible for air pollution and acid rain. Further investigations are necessary to quantify the contribution of wastewater treatment plants to these gases in the atmosphere and to evaluate measures to be taken to prevent such gas emissions.

SUMMARY

Phosphate emission into surface waters can be reduced by treating sewage in wastewater treatment plants which are run alternately anaerobic and aerobic. Under these conditions, sludge in wastewater treatment plants becomes enriched with polyphosphate accumulating bacteria. Phosphate is released by the sludge under anaerobic conditions. During aerobiosis, this amount of phosphate is taken up by the sludge together with phosphate from the influent, resulting in very low phosphorus concentrations in the effluent of wastewater treatment plants. The aim of the research presented in this thesis was to obtain more knowledge about the complex bacterial interactions within the activated sludge process removing biologically phosphate. A simple system was developed, in which biological phosphate removal could be studied under well defined conditions. The so-called fill and draw system consists of a one reactor vessel in which sludge is fed intermittently with a chemically defined medium containing acetate and glucose as carbon sources. The sludge was subjected to cycles with an anaerobic period, an aerobic period and a period allowing the sludge to settle. The resulting sludges were extremely enriched with polyphosphate accumulating bacteria. The phosphorus content reached up to 110 mg P/g dry weight. The amount of polyphosphate in the cells during steady state depended on the acetate:glucose ratio, the nitrate, and phosphate concentration in the medium. Highest phosphate accumulation was obtained with an acetate:glucose ratio of 9:1. Intracellular polyphosphate was formed during the aerobic period and was anaerobically hydrolysed and released as phosphate into the medium. In the absence of oxygen and in the presence of 2 g acetate-COD/l, 80-90% of phosphate was released by sludge containing 100 mg P/g dry weight. In the absence of acetate only 2-19% of the accumulated phosphate was excreted. The amount of precipitated metal phosphates and the acid soluble polyphosphate concentration were measured in several sludges and pure cultures of

Acinetobacter strains. The percentage metal phosphates of the total phosphorus content of the sludges varied between negligible and about 80%. Sludges from fill and draw systems with over 100 mg P/g dry weight, were almost completely devoid of these precipitates. The same was true for polyphosphate-accumulating cultures of *Acinetobacter*. Besides high molecular weight polyphosphate, the sludges contained also low polymeric polyphosphates (LPP), ranging from 0 to 50% of the total phosphate content. The LPP fraction in polyphosphate accumulating cultures of *Acinetobacter* strains was between 3 and 23% of their total phosphorus content. Sludges developed in the fill and draw systems could release in the presence of acetate more than 50% of the accumulated phosphate within 60 min. About 66% of this phosphate originated from LPP. Pure cultures of *Acinetobacter* strains released in the same time less than 1 mg P/g dry weight. The initial rate of phosphate release by sludge of a wastewater treatment plant designed to remove biologically phosphate (Renpho sludge) in the presence of acetate was reduced by addition of nitrate. In the presence of 16 mM acetate, maximum inhibition of 40 to 50% occurred at a nitrate concentration of 3.6 mM. Increasing the amount of acetate 10 times above the theoretical amount needed for complete denitrification, did not overcome the effect of nitrate. After depletion of nitrate, the phosphate release rate recovered slightly. In the presence of KCN and azide, inhibitors of denitrification, nitrate did not inhibit phosphate release. After completion of phosphate release and with sufficient acetate present, phosphate was taken up after addition of 3.6 mM nitrate. This phosphate uptake was too small to explain the reduction of the initial phosphate release rate, when nitrate was added at the start of the experiments. The formation of precipitated phosphates by an increase of the pH in the medium as a result of denitrification could only partly explain the reduction of the initial phosphate release rate. Nitrous oxide added at a concentration of 3.6 mM in the liquid phase had no negative effect on the phosphate release. Nitrite showed about the same effect as nitrate.

Nitric oxide at a constant partial pressure inhibited phosphate release by 50% at a concentration of 30 μM. The inhibitory effect of nitric oxide on the phosphate release was slightly reduced by addition of 5 mM KCN. Nitric oxide production by sludges removing biologically phosphate was dependent on the sludge type, the nitric oxide precursor, the carbon source and the pH. At pH 7.5, rates of nitric oxide production by Renpho sludge were 390 μmol/g sludge dry weight.h upon incubation with acetate plus nitrite, 16 μmol/g dry weight.h with acetate plus nitrate and 25 μmol/g dry weight.h with glucose plus nitrate. The highest rate of nitric oxide production upon incubation with acetate plus nitrite was 550 μmol/g dry weight.h at pH 8. At pH 7.5, rates of nitric oxide production by sludge from the fill and draw systems were 100-160 μmol/g dry weight.h upon incubation with acetate plus nitrite, 6-7 μmol/g dry weight.h, with acetate plus nitrate and 13-16 μmol/g dry weight.h with glucose plus nitrate. Pasteurized sludge produced nitric oxide only upon incubation with nitrite (12 μmol/g dry weight.h). The results show that nitric oxide is formed by biological processes in sludges from wastewater treatment plants removing biologically phosphate and that in the systems tested nitric oxide is responsible for the reduced phosphate release under denitrifying conditions.

SAMENVATTING

De fosfaatemissie op oppervlaktewateren kan gereduceerd worden door rioolwater te behandelen in afvalwaterzuiveringsinrichtingen (AWZI's), die afwisselend anaëroob en aëroob bedreven worden. Door deze bedrijfsvoering wordt het slib verrijkt met polyfosfaatophopende bacteriën. Onder anaërobe omstandigheden geeft het slib fosfaat af, terwijl bij aëroob bedrijf deze hoeveelheid fosfaat weer door het slib wordt opgenomen tezamen met fosfaat uit het rioolwater. Dit resulteert in lage fosfaatconcentraties in het effluent van deze AWZI's. Het doel van het onderzoek dat wordt beschreven in dit proefschrift, was om meer kennis te verkrijgen in de complexe bacteriële interacties van het actiefslibproces met biologische fosfaatverwijdering. Hiertoe werd een eenvoudig systeem ontwikkeld, waarin de biologische fosfaatverwijdering onder goed gedefinieerde condities bestudeerd kon worden. Dit zgn. fill and draw-systeem bestond uit één reactorvat met slib dat intermitterend gevoed werd met een kunstmatig samengestelde voeding met acetaat en glucose als koolstofbronnen. Het slib werd onderworpen aan cycli met anaërobe, aërobe en bezinkingsperioden. Dit resulteerde in slibsoorten die extreem verrijkt waren met polyfosfaat-ophopende bacteriën. Een fosfaatgehalte tot 110 mg P/g drogestof werd bereikt. Tijdens "steady state" condities was de hoeveelheid geaccumuleerd fosfaat in de cellen afhankelijk van de acetaat:glucose-verhouding, de nitraatconcentratie, en de fosfaatconcentratie in de voeding. Het hoogste fosfaatgehalte werd gevonden bij een acetaat:glucose-verhouding van 9:1. Intracellulair polyfosfaat werd gedurende de aërobe periode gevormd en werd tijdens de anaërobe periode gehydrolyseerd en afgegeven als fosfaat in het medium. In afwezigheid van zuurstof en in aanwezigheid van 2 g acetaat-CZV/l werd door slib met een fosfaatgehalte van 100 mg P/g drogestof 80-90% van het fosfaat afgegeven. In afwezigheid van acetaat werd slechts 2-19% van het geaccumuleerde fosfaat afgegeven. In verschillende soorten slib en reinculturen van

Acinetobacter stammen werden de hoeveelheden neergeslagen metaalfosfaten en zuuroplosbare polyfosfaten bepaald. Het percentage metaalfosfaten van het totale fosforgehalte van de soorten slib varieerde van te verwaarlozen tot ongeveer 80%. Metaalfosfaten waren nauwelijks aanwezig bij slib met meer dan 100 mg P/g drogestof afkomstig uit de fill and draw-systemen. Hetzelfde gold voor polyfosfaat-accumulerende reinculturen van *Acinetobacter*. Naast hoog polymeer polyfosfaten bevatten de soorten slib ook laag polymeer polyfosfaten (LPP). De hoeveelheid LPP varieerde van 0 tot 50% van het totale fosforgehalte. De LPP-fractie in polyfosfaat-accumulerende reinculturen van *Acinetobacter*-stammen varieerde van 3 tot 23% van het totale fosforgehalte. Slib dat zich ontwikkeld had in de fill and draw-systemen gaf met acetaat binnen 60 min meer dan 50% van het geaccumuleerde fosfaat af. Ongeveer 66% van deze hoeveelheid fosfaat was afkomstig van LPP. Reinculturen van *Acinetobacter* stammen gaven in deze periode minder dan 1 mg P/g drogestof af. De initiële snelheid, waarmee fosfaat door slib uit een AWZI met biologische fosfaatverwijdering (Renpho-slib) in aanwezigheid van acetaat werd afgegeven, werd door toevoeging van nitraat gereduceerd. Een maximale remming van 40 tot 50% in aanwezigheid van 16 mM acetaat vond plaats bij een nitraatconcentratie van 3,6 mM. Verhoging van de hoeveelheid acetaat tot boven 10 keer de theoretische hoeveelheid, die nodig is voor complete denitrificatie, maakte het effect van nitraat niet ongedaan. Na volledige omzetting van nitraat nam de fosfaatafgiftesnelheid enigszins toe. Nitraat remde de fosfaatafgifte niet in aanwezigheid van de remmers van de denitrificatie, KCN en azide. Na beëindiging van de afgifte en met nog voldoende acetaat aanwezig, werd na toevoeging van 3,6 mM nitraat fosfaat opgenomen. Deze fosfaatopname was evenwel te beperkt voor het verklaren van de reductie van de initiële fosfaatafgiftesnelheid bij toevoeging van nitraat aan het begin van de experimenten. De vorming van neergeslagen fosfaten door een stijging van de pH in het medium als een gevolg van denitrificatie kon slechts gedeeltelijk de reductie van de fosfaatafgifte verklaren. Lachgas, toegevoegd in een concen-

tratie van 3,6 mM aan de vloeistoffase, had geen remmend effect op de fosfaatafgifte. Nitriet had ongeveer hetzelfde effect als nitraat. Bij continue doorleiding van stikstofmonoxide werd de fosfaatafgifte voor 50% geremd bij een concentratie van 30 µM stikstofmonoxide. Het remmende effect van stikstofmonoxide op de fosfaatafgifte werd in geringe mate onderdrukt door de toevoeging van 5 mM KCN. De vormingssnelheid van stikstofmonoxide door biologisch fosfaat verwijderend slib was afhankelijk van het soort slib, het stikstofoxide waarmee geïncubeerd werd, de koolstofbron en de pH. De vormingssnelheden van stikstofmonoxide door Renpho-slib waren bij pH 7,5 390 µmol/g drogestof.h gedurende incubatie met acetaat plus nitriet, 16 µmol/g drogestof.h met acetaat plus nitraat en 25 µmol/g drogestof.h met glucose plus nitraat. De hoogste vormingssnelheden van stikstofmonoxide tijdens de incubaties met acetaat en nitriet werden gemeten bij pH 8 (550 µmol/g drogestof.h). De vormingssnelheden van stikstofmonoxide door slib uit de fill and draw-systemen bedroegen bij pH 7,5 100-160 µmol/g drogestof.h tijdens incubatie met acetaat plus nitriet, 6-7 µmol/g drogestof.h met acetaat plus nitraat en 13-16 µmol/g drogestof.h met glucose plus nitraat. Gepasteuriseerd slib produceerde alleen stikstofmonoxide tijdens incubatie met nitriet (12 µmol/g drogestof.h). De resultaten laten zien dat stikstofmonoxide gevormd kan worden door biologische processen in slib uit AWZI's en dat in de geteste systemen stikstofmonoxide verantwoordelijk kan zijn voor de gereduceerde fosfaatafgifte onder denitrificerende condities.

NAWOORD

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CURRICULUM VITAE

De auteur van dit proefschrift werd op 23 november 1952 geboren te Amsterdam. In 1971 werd aan de Osdorper Scholengemeenschap te Amsterdam het HAVO-diploma gehaald. Het diploma voor Botanisch analist werd in 1974 verkregen bij de school voor hoger beroepsonderwijs voor laboratoriumpersoneel te Amsterdam van de Amsterdamse Vereniging voor Opleiding van Scheikundig Hulppersoneel. Na een jaar als analist werkzaam geweest te zijn bij de Gemeente Waterleidingen van Amsterdam, werd begonnen met de studie Biologie aan de Vrije Universiteit te Amsterdam. Het doctoraalpakket bestond uit een hoofdvak Microbiologie, een uitgebreid bijvak Plantenfysiologie en een bijvak Milieukunde. Het laatste bijvak werd verricht aan de Universiteit van Amsterdam. Tijdens de studie werd als analist gewerkt bij de Gemeente Waterleidingen van Amsterdam en bij de afdeling Dermatologie van het Binnengasthuis te Amsterdam. Na het afstuderen in mei 1984 heeft de auteur een periode gewerkt bij de afdeling Microbiologie van de Vrije Universiteit te Amsterdam. Onderzoek werd verricht naar de groeiopbrengsten van een aantal streptomycine resistente mutanten van *Escherichia coli*. In december 1985 werd gestart bij de vakgroep Microbiologie van de Landbouwuniversiteit te Wageningen met onderzoek naar de biologische fosfaatverwijdering uit afvalwater, hetgeen uiteindelijk heeft geresulteerd in dit proefschrift. Sinds oktober 1990 is de auteur werkzaam bij BKH Adviesbureau te Delft als procestechnoloog en microbioloog.