Denitrification by heterotrophic bacteria during activated sludge treatment

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Abstract

It is generally accepted that *Pseudomonas* spp. are the predominant heterotrophic bacteria involved in denitrification during activated sludge treatment. However, uncertainty still exists regarding other bacteria involved. This study therefore aimed to determine which heterotrophic bacteria present in mixed liquor samples from a biological nutrient removal process are responsible for denitrification as well as to establish the extent to which these bacteria contribute to nitrate and nitrite reduction under anoxic conditions. Heterotrophic bacteria were isolated, using plating techniques, from the anoxic zone of the Darvill activated sludge process and assessed for nitrate and nitrite reduction under anoxic conditions. Results show a significant involvement of *Pseudomonas* spp. in nitrate and nitrite reduction. It was also found that many other heterotrophic bacteria are involved to some extent in denitrification, most of which were found to be incomplete denitrifiers only capable of reducing nitrates to nitrites with no further reduction of the nitrites produced. Furthermore, results demonstrated varying strengths of nitrate and nitrite reduction amongst the isolated heterotrophic bacteria, possible simultaneous oxygen and nitrate respiration by many incomplete denitrifiers as well as involvement of gram-positive rods and gram-negative cocci.

Introduction

The activated sludge process is the most widely applied biological wastewater treatment process in the world. Originally the process was designed as a single aerobic reactor for the removal of organic matter from wastewater but it has since been significantly developed to enhance its nutrient removal capabilities (Lu and Leslie Grady (Jr), 1988; Gray, 1990; Ekama et al., 1992; Wentzel et al., 1992). These improvements were induced by modifying the process from a single aerobic reactor to multi-reactor processes consisting of anaerobic, anoxic and aerobic zones with inter-reactor recycles thus enabling the process to progressively include nitrification, denitrification and phosphorus removal (Wentzel et al., 1992). These nitrification/denitrification/biological excess phosphorus removal processes are referred to as biological nutrient removal (BNR) processes and are currently being designed and implemented worldwide using established mathematical models and related software (Gujer and Kappler, 1992). These models provide very accurate information regarding process design and performance and can result in the development or simulation of effective BNR processes. However, according to Henze (1992) and Kristensen et al. (1992), these activated sludge models fall short in that they do not take into consideration the structure of biomass present in the process. Success of an activated sludge process is ultimately dependent on the functions of the constituent micro-organisms as well as the related process parameters (e.g. anaerobiosis, anoxia, aerobiosis) affecting microbial growth and activity (Simpkin, 1988; Bux et al., 1994). It is therefore believed that inadequate control of the micro-organisms in the activated sludge process is responsible for many variations in process performance. This is due to a lack of understanding of the ecological, physiological and biochemical activities of these micro-organisms which is resulting in growing movement towards a better understanding in order to gain optimal control of the process (Lu and Leslie Grady Jr, 1988; Davelaar, 1989; Wagner et al., 1993; Jansen et al., 1994; Hu et al., 1996; Satoh et al., 1996; Hippen et al., 1997).

Denitrification by heterotrophic bacteria in activated sludge treatment is of particular interest in that nitrates and nitrites are eutrophic (Gray, 1990), hazardous to human health (Terblanche, 1991; Kempster et al., 1997) as well as inhibit phosphorus removal during activated sludge treatment (Gruenebaum and Dorgeloh, 1992; Kuba et al., 1996). Furthermore, denitrifying heterotrophic bacteria are often implicated in enhanced biological phosphorus removal (EBPR) both under aerobic as well as anoxic conditions (Osborn et al., 1989; Kuba et al., 1993; Kavanaugh and Randall, 1994; Jørgensen and Pauli, 1995; Kuba et al., 1997;). In a BNR process denitrification is achieved in the anoxic zone/s of the process. Under anoxic conditions certain heterotrophic bacteria are stimulated into utilising nitrates and nitrites as final electron acceptors for cellular respiration in place of oxygen (Ketchum, 1988; Cappuccino and Sherman, 1992). This results in oxidation of organic matter as well as reduction of the nitrates and nitrites into nitrous oxides and nitrogen gas (Wanner and Grau, 1988).

In the wastewater industry uncertainty exists regarding the bacteria involved in denitrification as well as the extent to which these bacteria contribute to nitrate and nitrite reduction under anoxic conditions. It is generally presumed that *Pseudomonas* spp., as well as being involved in EBPR (Osborn et al., 1989; Kavanaugh and Randall, 1994; Jørgensen and Pauli, 1995), are the predominant micro-organisms through which denitrification is achieved (Janda et al., 1988; Gray, 1990; Lazarova et al., 1992). According to Otlanabo (1993) various species of *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Flavobacterium*, *Hyphomicrobium*, *Pseudomonas*, *Vibrio* and others are responsible for denitrification in soil. It therefore seems unlikely that only *Pseudomonas* spp. are responsible for denitrification occurring in such an incredibly diverse microbial consortia as that of activated sludge.

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Denitrification is generally accepted as being the reduction of nitrates, via nitrites, to nitrous oxides and nitrogen gas. However, many denitrifying bacteria only have the enzymic ability to reduce nitrates to nitrites with no further reduction of the nitrites produced (Rheinheimer, 1985; Ketchum, 1988; Cappuccino and Sherman, 1992; Robertson and Kuenen, 1992; Glass et al., 1997). In fact, according to Rheinheimer (1985) and Robertson and Kuenen (1992), most of the denitrifying bacteria in aquatic systems are only capable of incomplete denitrification. The full effect of this on nitrogen removal during activated sludge treatment has, however, not yet been determined. A more intensive understanding of denitrifying heterotrophic bacteria is therefore essential as this may be one of the next steps to optimising efficiency of nitrogen and phosphorus removal in nutrient removal activated sludge processes.

The aim of this study was therefore to isolate and identify as many denitrifying heterotrophic bacteria as possible from the anoxic zone of an existing BNR process in order to establish the significance of *Pseudomonas* spp. in denitrification as well as to determine which other heterotrophic bacteria present in the process contribute to denitrification under anoxic conditions. In addition, to determine the extent to which each of the isolated denitrifying heterotrophic bacteria contribute to nitrate and nitrite reduction under anoxic conditions.

Experimental

Isolation of heterotrophic bacteria

Three random mixed liquor samples were taken from the anoxic zone of the BNR process at Darvill Wastewater Works. Samples were homogenised using glass beads to facilitate isolation of the heterotrophic bacteria. Triplicate serial dilutions (10^{-1} to 10^{-8}) were made from each sample and plated onto both casitone glycerol yeast autolysate agar (CGYA) and heterotrophic plate count agar (mHPC) (Difco Laboratories, USA) using the spread plate technique. CGYA is believed to be the most widely and effectively applied culture medium for isolating heterotrophic bacteria from activated sludge (Osborn et al., 1989; Gray, 1990; Bux et al., 1994) while mHPC has been developed for isolation of heterotrophic bacteria from soil and water samples (Bridson, 1995). All plates were incubated at 20°C for 5 to 7 d (Lötter and Murphy, 1985; Venter et al., 1989) whereafter heterotrophic bacteria were isolated.

Screening for nitrate and nitrite reduction

Monocultures of all heterotrophic bacteria isolated were screened in triplicate for nitrate and nitrite reduction by growing them in tubes of nitrate media (Merck, Germany) at 20°C for 5 to 7 d. The nitrate medium used was supplemented with 0.1% agar in order to semi-solidify it, thus impeding oxygen diffusion and creating anoxic conditions (Cappuccino and Sherman, 1992). After incubation all tubes were analysed to assess growth of the isolated bacteria under anoxic conditions whereafter nitrate and nitrite reduction capabilities of each isolate were assessed using the nitrate reduction test proposed by Cappuccino and Sherman (1992). Incubation temperatures were maintained at 20°C throughout the study.

Identification of heterotrophic bacteria

After nitrate and nitrite reduction screening, heterotrophic bacteria that could reduce nitrates and nitrites were identified using Gram stains, API 20E, API 20NE, key differential biochemical tests and morphological characteristics. All heterotrophic bacteria identified were identified to at least generic level. Gram-negative rods were identified using API 20E and API 20NE, as described by Bux et al. (1994), in conjunction with additional key differential biochemical tests while gram-positive rods and gram-negative cocci were identified using key differential biochemical tests and morphological characteristics (*Bergey's Manual*, 1984; 1986; Cappuccino and Sherman, 1992). However, numerous gram-negative rods were unidentifiable, even with repeated identification tests using API identification kits. It is possible that the database for API identification kits is insufficient for application to activated sludge studies owing to the very diverse consortia of bacteria present and possible occurrence of mutants. Furthermore, not all gram-positive rods were identified. This was due to difficulty in establishing sufficient key differential biochemical tests to effectively distinguish amongst the wide array of possible denitrifying gram-positive rods known to be present in the environment. Not enough is known about the presence and role of gram-positive bacteria in activated sludge treatment and a more efficient means is required for identification and assessment of these micro-organisms in the future. No denitrifying gram-positive cocci were isolated for identification.

Results and discussion

True denitrifiers

The general perception throughout the wastewater industry is that, as well as being implicated in EBPR, *Pseudomonas* spp. are the predominant micro-organisms responsible for denitrification occurring during activated sludge treatment. It is also presumed that this genera of micro-organisms are responsible for complete reduction of nitrates, via nitrites, to nitrous oxides and nitrogen gas. However, in confirmation with findings by Glass et al. (1997), the results of this study showed the presence of two different types of

TABLE 1
TRUE DENITRIFYING HETEROTROPHIC BACTERIA SHOWING STRONG REDUCTION OF BOTH NITRATES AND NITRITES

Identity	Morphology	Gram stain
Achromobacter xylosoxidans	Rod	-ve
Pasteurella spp.	Rod	-ve
Pseudomonas aeruginosa	Rod	-ve
Pseudomonas pickettii	Rod	-ve
Pseudomonas stutzeri	Rod	-ve
**Pseudomonas testosteroni/alcaligenes*	Rod	-ve
Unidentified bacterium A (possibly a strain of *Pseudomonas fluorescens 2*)	Rod	-ve

* A doubtful identification using API identification kits.

denitrifiers in the Darvill BNR process. The first of these were true denitrifiers i.e. bacteria that were able to reduce both nitrates as well as nitrites (Tables 1, 2 and 3). *Pseudomonas* spp. predominated amongst the true denitrifiers. However, it was seen that reduction of nitrates and nitrites is also carried out by other heterotrophic bacteria in the process. Results show that seven different species of *Pseudomonas*, namely *P. aeruginosa*, *P. fluorescens 1*, *P. fluorescens 2*, *P. mallei*, *P. pickettii*, *P. stutzeri*, *P. testosteroni/alcaligenes* are, along with *Achromobacter xylosoxidans*, *Flavobacterium indologenes*, *Pasteurella spp.*, unidentified bacterium A and unidentified bacterium B, responsible for nitrate as well as nitrite reduction occurring under anoxic conditions.

TABLE 2
TRUE DENITRIFYING HETEROTROPHIC BACTERIA SHOWING WEAK REDUCTION OF NITRATES AND NITRITES

Identity	Morphology	Gram stain
Pseudomonas fluorescens 2	Rod	-ve
**Pseudomonas mallei*	Rod	-ve
Unidentified bacterium B	Rod	-ve

* A doubtful identification using API identification kits.

TABLE 3
TRUE DENITRIFYING HETEROTROPHIC BACTERIA SHOWING INCONSISTENT REDUCTION OF NITRITES

Identity	Morphology	Gram stain
Flavobacterium indologenes	Rod	-ve
Pseudomonas fluorescens 1	Rod	-ve

TABLE 4
INCOMPLETE DENITRIFYING *PSEUDOMONAS* SPP. SHOWING STRONG REDUCTION OF NITRATES TO NITRITES

Identity	Morphology	Gram stain
Pseudomonas acidovorans	Rod	-ve
Pseudomonas cepacia	Rod	-ve
Pseudomonas fluorescens 1 - strains 1 - 3	Rod	-ve
**Pseudomonas fluorescens 1* - strain 4	Rod	-ve
Pseudomonas fluorescens 3 - strains 1 - 2	Rod	-ve
**Pseudomonas luteola*	Rod	-ve
Pseudomonas maltophia	Rod	-ve
**Pseudomonas mendocina*	Rod	-ve
Pseudomonas pickettii - strains 1 - 2	Rod	-ve
**Pseudomonas pickettii* - strain 3	Rod	-ve
Pseudomonas stutzeri - strain 1	Rod	-ve
**Pseudomonas stutzeri* - strain 2	Rod	-ve
Pseudomonas tostesteroni/alcaligenes - strains 1 - 5	Rod	-ve
**Pseudomonas tostesteroni/alcaligenes* - strain 6	Rod	-ve
Pseudomonas vesicularis	Rod	-ve
Pseudomonas spp. (i - vi)	Rod	-ve

* A doubtful identification using API identification kits.

This study also revealed that some true denitrifying bacteria cannot reduce nitrates and nitrites as effectively as others (Table 2). *P. aeruginosa*, *P. pickettii*, *P. stutzeri*, *P. testosteroni/alcaligenes*, *Achromobacter xylosoxidans*, *Pasteurella* spp. and unidentified bacterium A all proved to reduce nitrates and nitrites completely within the allocated incubation period whereas *P. fluorescens 2*, *P. mallei and* unidentified bacterium B could not completely reduce all the nitrates and nitrites. This could possibly be attributed to slow growth rate or weak nitrate/nitrite reductases. It was, however, noted that, with *P. fluorescens 2* and unidentified bacterium B, nitrite reduction seemed to proceed instantaneously as nitrites were produced from nitrate reduction. It appears that *P. fluorescens 2* and unidentified bacterium B contain weak nitrate reductases yet strong nitrite reductases. *P. mallei*, on the other hand, proved to reduce nitrates to nitrites very efficiently yet did not reduce all the nitrites produced from nitrate reduction. Unlike *P. fluorescens 2* and unidentified bacterium B it seems that *P. mallei* may only contain a weak nitrite reductase while having a strong nitrate reductase. Interesting results were also obtained concerning nitrate and nitrite reduction by *Flavobacterium indologenes* and *Pseudomonas fluorescens 1* (Table 3). Both bacteria only reduced nitrates to nitrites and no further but would on some occasions reduce nitrites as well. The reason behind this is uncertain but it is thought that it may be due to genotypic variations amongst some of the bacterial cells as no contamination by other bacteria was apparent.

Another interesting observation was a possible relationship between unidentified bacterium A and the weaker denitrifying *P. fluorescens 2*. Unidentified bacterium A was not identified due to the fact that it seemed to be non-viable by the time identification was possible. However, it was noted that repeated sub-culturing of the bacterium always resulted in increasing numbers of *P. fluorescens 2* and decreasing numbers of unidentified bacterium A. This observation was made throughout the duration of the study and it is thought that unidentified bacterium A may have possibly been a mutant of the *P. fluorescens 2* strain which was simply reverting through repeated sub-culturing. It is possible that under the stressful conditions imposed on the microorganisms during activated sludge treatment that mutations may occur in order to enable the cells to cope with the harsh conditions.

Results of this study also show that there are no gram-positive rods/cocci or gram-negative cocci in the Darvill BNR process which can reduce nitrates as well as nitrites. However, the absence of true denitrifying gram-positive heterotrophic bacteria may be questionable in that a study done by Wagner et al. (1994) showed, with the use of molecular techniques, that although gram-negative heterotrophic bacteria are predominant in activated sludge, gram-positive heterotrophic bac-

TABLE 5
INCOMPLETE DENITRIFYING HETEROTROPHIC BACTERIA SHOWING STRONG REDUCTION OF NITRATES TO NITRITES

Identity	Morphology	Gram stain
**Achromobacter* group VD	Rod	-ve
Achromobacter xylosoxidans	Rod	-ve
Aeromonas hydrophila - strains 1 - 2	Rod	-ve
Aeromonas salmonicida 2 - strains 1 - 3	Rod	-ve
Agrobacterium radiobacter	Rod	-ve
**Alcaligenes denitrificans*	Rod	-ve
Cedecia spp.	Rod	-ve
**Chromobacterium violaceum*	Rod	-ve
Citrobacter fruendii	Rod	-ve
**Enterobacter agglomerans 3*	Rod	-ve
Enterobacter intermedium	Rod	-ve
Escherichia coli 1	Rod	-ve
Escherichia coli 2	Rod	-ve
Klebsiella pneumoniae ssp. *pneumoniae*	Rod	-ve
Klebsiella oxytoca 1 - strain 1	Rod	-ve
**Klebsiella oxytoca 1* - strain 2	Rod	-ve
Moraxella phenylpyruvica - strains 1 - 2	Rod	-ve
Moraxella spp.	Rod	-ve
**Moraxella spp.*	Rod	-ve
Pasteurella multocida	Rod	-ve
Pasteurella pneumotropica	Rod	-ve
Pasteurella spp.	Rod	-ve
**Rahnella aquatilis*	Rod	-ve
Serratia liquifaciens - strains 1 - 3	Rod	-ve
Serratia marcescens	Rod	-ve
**Vibrio damsela* -strains 1 - 2	Rod	-ve
Vibrio fluvialis	Rod	-ve
**Vibrio parahaemoliticus*	Rod	-ve
Yersinia intermedium	Rod	-ve
**Unidentified bacteria C - M	Rod	-ve
Bacillus spp.	Rod	+ve
****Unidentified bacteria N - T	Rod	+ve
Neisseria spp. (i - iv)	Coccus	-ve
****Neisseria* spp. v (Possibility of *Bramhamella ovis)*	Coccus	-ve

* A doubtful identification using API identification kits.
** An inconclusive identification using API identification kits.
*** A doubtful identification using key differential biochemical tests.
**** No identification due to lack of key differential biochemical tests.

teria may be playing a greater role than previously believed. Wagner et al. (1994) demonstrated that the use of enriched media actually selects against gram-positive heterotrophic bacteria and it is therefore possible that the results of this study may in fact be somewhat biased in connection with the presence of gram-positive denitrifying bacteria in the Darvill BNR process.

Incomplete denitrifiers

The results show that most of the denitrifying heterotrophic bacteria isolated were incomplete denitrifiers i.e. bacteria that were only capable of reducing nitrates to nitrites with no further reduction of the nitrites produced (Tables 4, 5 and 6). These results substantiate findings by Rheinheimer (1985), Robertson and Kuenen (1992) and Rosén and Welander (1994). Robertson and Kuenen (1992) state that these incomplete denitrifying bacteria lack key nitrite reductase enzymes which enable complete denitrifiers to reduce nitrites. Complete reduction of nitrates to nitrous oxides and nitrogen gas under anoxic conditions during activated sludge treatment is actually being achieved via interactive associations between complete and incomplete denitrifying bacteria. Furthermore, it was found that a substantial proportion of the incomplete denitrifiers were *Pseudomonas* spp. thus further substantiating the significance of *Pseudomonas* spp. in denitrification (Table 4). However, results also show the presence of many other incomplete denitrifying bacteria in the Darvill activated sludge (Tables 5 and 6).

Other results of this study show that some of the incomplete denitrifiers showed weaker reduction of nitrates than others in that they did not reduce all the nitrates in the nitrate media into nitrites (Table 6). These bacteria either have slow growth rates or weak nitrate reductases in their cytoplasm. This, together with the absence of nitrite reductases, results in the production of small amounts of nitrites which accumulate gradually. It is evident, however, that there are many more strong nitrite-producing bacteria in the Darvill activated sludge than weak nitrite-producing bacteria (Tables 5 and 6).

Rheinheimer (1985), Robertson and Kuenen (1992), Boehler et al. (1994), Carter et al. (1995) and Hippen et al. (1997) offer findings that support claims of denitrifying bacteria being capable of simultaneous utilisation of nitrates and oxygen as terminal electron acceptors for cellular respiration. Robertson and Kuenen (1992) even report of a denitrifying bacterium which was unable to grow under proper anoxic conditions and reduced nitrates micro-aerophilically. Observations made in this study tend to confirm these findings in that most of the incomplete denitrifying bacteria, when tested for nitrate reduction, grew predominately in the more oxic regions of the nitrate media. It was seen that these bacteria preferred oxic conditions for growth but were still able to produce nitrites from nitrate respiration while simultaneously utilising oxygen as a final electron acceptor. These bacteria did not grow very well in oxygen limited regions of the nitrate media. It is therefore possible that these incomplete denitrifiers are reducing some nitrates to nitrites in the aerobic zone as well as in the anoxic zone of the process. Mauret et al. (1996) reported nitrite build-ups occurring in certain activated sludge processes and it was theorised that this nitrite accumulated as a result of incomplete nitrification in the aeration basin. However, it is possible that, in some instances,

TABLE 6
INCOMPLETE DENITRIFYING HETEROTROPHIC BACTERIA SHOWING WEAK REDUCTION OF NITRATES TO NITRITES

Identity	Morphology	Gram stain
**Unidentified bacterium U (Possibility of *Pasteurella* spp.)	Rod	-ve
**Unidentified bacterium V	Rod	-ve
****Unidentified bacterium W	Rod	+ve

** An inconclusive identification using API identification kits.
**** No identification due to lack of key differential biochemical tests.

these nitrite build-ups may have occurred due to the presence of large numbers of incomplete denitrifiers. According to Rheinheimer (l985) and Robertson and Kuenen (1992) initial nitrite production by actively denitrifying bacterial communities is usually very high and may, especially if accompanied by high initial nitrate concentrations, result in nitrite build-up. If the amount of incomplete denitrifiers grossly outnumber the complete denitrifiers and the nitrite oxidisers, i.e. *Nitrobacter* spp. and *Nitrospira spp.,* nitrite build-ups may occur as it is only these micro-organisms that possess the ability to reduce or oxidise the nitrites produced by incomplete denitrification. However, in a BNR process experiencing good nitrification it is most likely, considering sufficient oxygen is available to the nitrite oxidisers, that nitrites produced in the aerobic zone/s by incomplete denitrification are reoxidised to nitrates hence reducing the occurrence of nitrite build-up. It is still uncertain whether true denitrifiers possess any capacity for aerobic denitrification and can contribute to reduction of nitrates and nitrites in the aerobic zone/s of BNR processes.

Further results of this study also show the presence of incomplete denitrifying gram-positive rods as well as gram-negative cocci in the Darvill activated sludge (Table 5). The presence of gram-positive heterotrophic bacteria offers some confirmation to findings by Wagner et al. (1994) who claim that gram-positive bacteria may be playing a bigger role in activated sludge treatment than previously believed. Gram-positive bacteria are believed to play a major role in EBPR in some BNR processes (Wagner et al., 1994). However, the bias incurred by nutrient rich isolation media is making the presence of gram-positive bacteria in activated sludge go by largely unnoticed. This may explain the predominance of gram-negative bacteria observed over gram-positive bacteria in this study. Alternatively, the absence of large numbers of gram-positive bacteria in the Darvill BNR process may be indicative of the poor EBPR experienced in the plant and the resultant need for chemical precipitation of phosphates.

Conclusions

Pseudomonas spp. have a significant involvement in denitrification occurring in the Darvill BNR process. However, many different heterotrophic bacteria contribute to denitrification occurring in the process, most of which are incomplete denitrifiers only capable of reducing nitrates to nitrites with no further reduction of the nitrites produced. Some of these denitrifying heterotrophic bacteria may contain weak nitrate and nitrite reductases, therefore only enabling them to reduce nitrates and nitrites slowly and weakly. Many incomplete denitrifiers may be capable of simultaneous respiration of oxygen and nitrates thus resulting in reduction of nitrates in the aerobic zone. Although incomplete, some gram-positive rods as well as gram-negative cocci also contribute to denitrification. It is apparent that complete reduction of nitrates, via nitrites, to nitrous oxides and nitrogen gas, in the Darvill BNR process, is being achieved via interactive associations between complete and incomplete denitrifying heterotrophic bacteria. However, owing to the diverse consortia, identification of all these bacteria is ineffectual using only API 20E and API 20NE identification kits with standard differential biochemical tests and therefore a more efficient means is required for identification of constituent bacteria.

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