Nitrate-reducing bacterial community in hypernutrified aquatic environments

THESIS

NITRATE-REDUCING BACTERIAL COMMUNITY IN HYPERNUTRIFIED AQUATIC ENVIRONMENTS

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NITRATE-REDUCING BACTERIAL COMMUNITY IN HYPERNUTRIFIED AQUATIC ENVIRONMENTS
過栄養な水環境に生息する硝酸塩還元細菌の研究

竹　内　準　一

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Contents

Preface

This thesis has been submitted in partial fulfilment of the requirements for obtaining a Doctor of Philosophy (PhD) degree, consists of the following five chapters:

In the 1st chapter, the past up to recent literatures were reviewed to evaluate the main research topic in this study. Dissimilatory nitrate reduction to ammonia by *E. coli* in a test tube was recognised more than 50 years ago in Japan, but its ecological significance had been ignored till 1978, at that time two biogeochemists discovered independently. Microbiological studies on this topic have been focused by researchers in the UK.

In the 2nd chapter, culture-based experiments using a wide variety of water samples were carried out in Tokyo, which was of a minor research interest since even the textbooks had often neglected the nitrate ammonification process. Fermentative bacteria such as *Enterobacter/Aeromonas* were found to be responsible for this pathway, which were a traditional notion confirmed by the previous results using estuarine sediments and this research based in Tokyo.

In the 3rd chapter, activated sludge samples fed with nitrate of an industrial origin were dealt with as a counterpart of hypernutrified estuarine sediments. The process was initially intended to remove phosphorus with an anoxic selector but it acted as the site for denitrification fed with nitrate. However, the *Enterobacter/Aeromonas* members tended to be scarce in activated sludge after enrichment culture; this was different from those using estuarine sediments previously studied.

In the 4th chapter, a culture-independent method was applied to hypernutrified estuarine sediment samples, covering from freshwater to marine habitats, targeting the *nrfA*, a functional gene cording for nitrate ammonification. Based on this investigation, habitat segregation of the *nrfA*-related sequences was observed in accordance with the environmental gradients in terms of vertical and spatial separations. Apart from the existing members known as nitrate ammonifiers, *i.e.*, enterobacteriaceae and sulphate reducers, the present results also suggested the *Bacteroides* and other unknown clusters as a possible agent catalysing nitrate ammonification in the sediment system, constantly keeping a nitrite pool minimum. This result was owing to the non-cultural technique that enables us to deal with aerobes and anaerobes seamlessly.

In the 5th chapter, a long-term ignored pathway, nitrate ammonification was once recognised as a dead-end of dissimilatory nitrate reduction but re-evaluated as the ammonia supplying route to make a possible linkage with the anammox-coupled nitrogen removal from the water, particularly in the nitrite accumulated estuarine sediments.

In the appendix, a research note was introduced as a result of microbiological investigation on the influence of human waste on greywater treatment carried out in Bangkok. This result suggested the nitrogen source was eventually from the atmosphere via fertilisers, food and human waste. Thus, excess nitrogen should be recycled based on the source separation policy such as "ecosan", and if it is not possible, it could be recycled through the biogeochemical cycles of the manmade wastewater treatment process and naturally-occurring self-purification systems such as mudflats and wetlands.

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* * *

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Before the Christmas season has come to Llangefni,
Juni

Abbreviations

-Environmental Microbiology:

AHB, aerobic heterotrophic bacteria
AOB, ammonia-oxidizing bacteria
CFU, colony-forming units
DNB, denitrifying bacteria
DNRA, dissimilatory nitrate reduction to ammonia
MLSS, mixed liquor suspended solids
MPN, most probable number
NOB, nitrite-oxidizing bacteria
PHB, poly-*β*-hydroxybutyrate
Poly-P, polyphosphate
SRB, sulphate-reducing bacteria

-Molecular Microbiology:

BSA, Bovine serum albumin
bp, base pairs
dNTP, deoxynucleoside-5′-triphosphate
EBI, European Bioinfomatics Institute
EDTA, ethylene diamine tetra acetic acid
EtBr, Ethidium bromide
kb, 10^3 (Kilo-) base
ORF, open reading frame
RO, reserve osmosis
SDS, sodium dodecyl sulphate
SRS, sequence retrieval system
Tris, tris (hydroxymethyl) amino methane
TRS, template suppression reagent
UV, ultraviolet
X-Gal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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Chapter 1

Introduction and background

1. Nitrogen cycle in the environment

1.1 Nitrogen cycle and the artificial options

Nitrogen as an inert gas, dinitrogen (N_2), distributes most abundantly in the earth's atmosphere to form a nitrogen reservoir in the biosphere. Actually, however, only a small amount of dinitrogen is biologically available for the primary production via nitrogen fixation. Since nitrogen as well as phosphorus are essential elements for primary production, these limited elements tend to be deficient both in terrestrial and aquatic ecosystems.

The biogeochemical nitrogen cycle shows a complicated feature as compared with carbon and sulphur cycles. There are a wide variety of microorganisms involve in each step for the transformation of nitrogen compounds in several oxidation states, ranging from organic nitrogen to nitrate under aerobic and anaerobic conditions. There consists of a set of metabolic pathways from one compound to another in this matter cycle so that the element can be recycled through biological activity. Take the translocation of nitrogen between the gas and liquid phases for example; there is a pair of opposite directions known as the nitrogen fixation and denitrification.

Human activities such as agriculture and sewage works increase the load of nitrogen, eventually from the nitrogen pool in the air to aquatic ecosystems, by applying fertilizers to the land and discharging sewage effluents to the receiving waters (Malone, 1984). These two major nitrogen loads are

basically originated from synthesized nitrogen produced via the Haber-Bosch's industrial process of nitrogen fixation and night soil (*i.e.* human waste) contamination via sewerage networks. While the positively charged ammonium is strongly absorbed to the negatively charged clay minerals and remained in soil, the negatively charged nitrate is easily leached from soil in farms and forests after rainfall. Apart from land runoff, secondary treated sewage also has high nitrate and ammonium contents, which are derived from human excreta and the other household wastewater.

1.2 Estuaries as a nitrate sink

Estuaries are generally characterised as the transition from freshwater to marine environments and a resulting trap for both particulate organic debris and clay particles flocculated there. These variable conditions make it possible for both halophilic and non-halophlic microorganisms to inhabit this specialised ecological niche. Suspended particles may cause the limitation of primary production but provide the habitat on which dense bacteria could attach. These environmental pressures might select versatile prokaryotic communities more adaptable to severe fluctuating conditions rather than eukaryotic communities such as protozoan and algae. Therefore, estuarine environments of high turbidity with resuspended particles are considered to be a naturally occurring biological reactor in this respect (Owens, 1986).

In addition, estuaries are often recognised to be a nitrogen sink having a strong environmental gradient of nitrogen loading of anthropogenic origins. Major sources of the nitrogen loading into the estuarine environment are land

runoff as a non-point source and sewage effluents as a point source of water pollution. As a result, estuaries act as the most active site of dissimilatory nitrate reduction and the subsequent denitrificaion, whose microbial reactions proceed under anoxic condition. Actually, aerobic nitrification and anoxic denitrification can simultaneously occur within the microhabitat of the upper sediment and possibly the detritus-rich water column (Rheinheimer, 1992).

Tidal river mouths also act as a filter in terms of attenuating matter flow along the estuaries (Malone, 1984). There are few differences between denitrification in freshwater and marine ecosystems but a distinct difference in the estuarine system from the formers. While autochtonous nitrate via nitrification in the sediment is the major source of substrate for denitrification in stagnant waters such as lakes and closed bays, a large amount of allochthonous nitrate loads from the outside in estuaries increases the denitrification rate linearly (Seitzinger, 1988). Nearly 50% of the dissolved inorganic nitrogen loads to estuaries were continuously lost by denitrification, which were often depending on the freshwater retention time within the tidal zone (Nedwell *et al.*, 1999). Thus, denitrification in estuaries has been proposed as a naturally occurring pollution control mechanism to restore eutrophic coastal environments.

Sewage effluent could stimulate and enrich the potential activities of microbial nitrate reduction and denitrification as a self-purification process in a mangrove estuary (Nedwell, 1975) and a freshwater lake (Jones *et al.*, 1980). Moreover, the possible anammox process (anaerobic ammonium oxidation), which was originally reported in a wastewater treatment module (Mulder *et al.*,

1995), might be also significant in removing both nitrite and ammonium in the specific location close to the effluent outfalls of sewage works in the Colne (Ogilvie *et al.*, 1997b) and the Thames (Trimmer *et al.*, 2000) estuaries. Nitrite is a minor component of inorganic nitrogen species in the water, but plays a central role as an intermediate in a local nitrogen flow (Dong *et al.*, 2002).

1.3 Sediment as the active site of microbial activity

The bottom sediments exceed the overlying water as the most active site for microbial degradation process (Nedwell *et al.*, 1999) in terms of the microbial abundance and activity. The majority of mineralisation by heterotrophic microorganisms and benthic primary production by microalgae occurs in the uppermost layer of sediments, forming a vertical zonation of aerobic, anoxic (or suboxic) and anaerobic microbial processes in the sediment profile.

Not only aerobic respiration but also anaerobic respiration contributes to the breakdown of organic carbon as an electron donor in aquatic sediments. A conceptual model of relative importance of microbial respiratory processes was well described by Capone and Kiene (1988). Nitrate and sulphate could be used as terminal electron acceptors by nitrate- and sulphate-reducing bacteria under anoxic and anaerobic conditions, respectively. Sulphate reduction predominates in the marine environment due to the limitless supply of sulphate from seawater, while methanogenesis predominates in the freshwater environment abundant in biodegradable organic matter. Although nitrate-reducing bacteria are widely distributed, their activity tends to be strictly limited by nitrate and organic inputs.

Nitrate concentrations in the pore water of sediment are usually lower than in the overlying water because benthic nitrate-reducing bacteria consume nitrate continually. Thus, nitrate-reducing bacterial communities in the sediment must be working below the optimal concentrations of ambient nitrate (Nedwell *et al*., 1999). Tidal invasion of seawater could also cause the dilution of nitrate available as an electron acceptor in the estuarine environments. Thus, the actual *in situ* rates of denitrification turned out negligible due to the insufficiency of nitrate, though the potential denitrifying populations inhabited in a salt marsh at the river mouth of the Colne estuary (Aziz and Nedwell, 1979). After all, estuarine sediments provide the most favourable habitat for the nitrate-reducing bacterial community, and the supply of nitrate would be a limiting factor for their activity.

2. Biogeochemical pathways of nitrate reduction

2.1 Two major fates of nitrate in costal sediments

Nitrate in the effluents discharged from sewage works is expected to be denitrified in sediments of the receiving waters. However, based on the geochemical studies of nitrate metabolism in sediments using tracer technique, nitrate was not always reduced to gaseous nitrogen such as dinitrogen and nitrous oxide via the denitrification but returned to ammonium remaining soluble in the water (Koike and Hattori, 1978). The latter process is now termed nitrate ammonification, which is an opposite direction of nitrification. In other words, the pathways and the subsequent end-products of dissimilatory nitrate reduction in sediments vary depending on the surrounding conditions.

According to Koike and Hattori (1978), dissimilatory ammonia and assimilatory organic nitrogen production were roughly correlated with the organic content of the sediment. Sørensen (1978) showed that the capacity for reduction of nitrate to ammonia was relatively significant in deeper layers of the sediment. These data suggest that nitrate ammonification is likely to be equally as important as denitrification, both competing for nitrite in the nitrogen cycle in the sediment system.

2.2 Ecological implications of nitrate reduction

These two fates of nitrate reduction, namely, denitrification and nitrate ammonification have quite different significance from the ecological viewpoint. Denitrification results in the complete removal of dissolved nitrogen to the atmosphere, while nitrate ammonification retains the nitrogen within the water environment. Thus, the former contributes to the removal of nitrogen from the water.

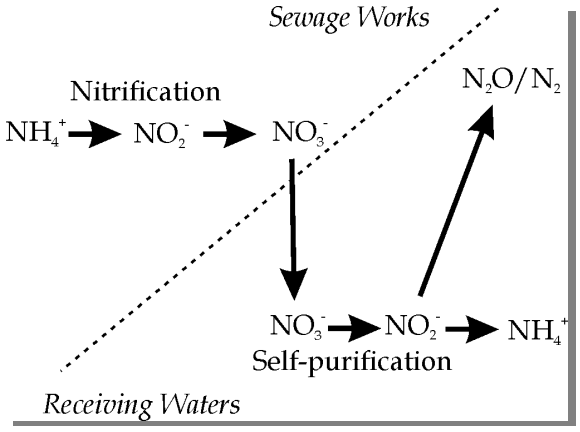


Figure 1-1 Nitrification in sewage works and self-purification in the receiving waters

Nutrient removal is one of the major concerns in wastewater treatment to prevent aquatic environments from eutrophication. In contrast with reliable phosphorus removal with chemical coagulants, nitrogen removal involves unstable biological reactions. Since a combination of nitrification and denitrification is mutually exclusive in terms of different oxidation-reduction potentials, plant designers and operators have been compelled to take these opposing requirements into account when employing a conventional two-stage reactor, consisting of carbonaceous waste removal and nitrogen removal (aerobic nitrification and anoxic denitrification) processes (Horan, 1990).

Now that novel concepts in the nutrient removal have been established, a single-stage reactor should be developed so as to pursue a small space and energy-saving operation (Robertson and Kuenen, 1992). The "nitrite-shunt" concept has been proposed as a combination of heterotrophic nitrification and denitrification from nitrite using *Thiophaera pantotropha* (Robertson and Kuenen, 1983; amended as *Paracoccus pantotrophus*), in which the specific species hardly settled in a full-scale plant of activated sludge microbial process. Later, the "anammox" module has alternatively been developed as a naturally-occurring microbial assemblage exhibiting a unique denitrification pathway from nitrite and ammonia. It will be most interesting to see whether these intensive microbial processes were also predicted in a natural habitat such as hypenutrified estuaries. The anammox-like phenomena were observed in the Colne and the Thames estuaries as cited above, however, its microbiological study remains to be investigated in the future. Originally, any wastewater treatment process substitutes the self-purification process in nature.

2.3 Microbial interactions in estuarine sediments

As contrasted with terrestrial soils, the appearance of submerged sediment materials looks so smooth that the microbial community structure might be rather homogeneous. However, on closer examination, it forms a complex assemblage consisting of a wide variety of autotrophic and heterotrophic microbial populations that affect each other in terms of commensalism and competition for necessary nutrients and the other environmental factors such as light intensity, oxidation-reduction potential and environmental temperature.

Microalgae often forming biofilms on the surface of a drained tidal flat could compete with nitrate reducing bacteria for inorganic nitrogen, whereas the nutrient uptake rates by the nitrate-reducing bacterial group could surpass those by the benthic algae in the submerged estuarine sediments under the water column of high turbidity. Diatoms as microphytobenthos assimilated only a few per cent of total inorganic nitrogen inputs into the Colne estuary (Dong *et al.*, 2000). However, microalgae could inhabit the uncoupled denitrification process (depending on external nitrate supply from overlying water) by photosysthetic oxygen diffusion, but could stimulate the coupled nitrification-denitrification process when depends on internal nitrate from sediment via nitrification. An and Joye (2001) reported that benthic photosynthesis in shallow estuarine sediments could enhance the coupled nitrification-denitrificatin when ammonium was not limiting.

Nitrification is mostly referred to as an aerobic lithotrophic process, converting ammonium to nitrate via nitrite. Since the optimum temperature for the growth of nitrifying isolates ranged from 25 to 35^{o}C, the growth rate

remarkably decline below 15°C. Hence environmental temperature and the subsequent oxygen solubility tend to affect the nitrification rates in sediments, often showing a seasonal and diurnal fluctuation patterns. It is now recognised that nitrification is a significant regulating factor in generating an essential substrate to the coupled nitrification-denitrification regime within estuarine sediments. Although nitrification activity could be inhibited by sunlight, it might be a minor factor that influences the nitrogen metabolism in heavily turbid estuarine waters.

Ammonification is a part of the microbial mineralisation process occurs extensively in estuarine sediments, which is the pathway opposing assimilatory nitrate reduction. Ammonification rates in vegetated sediments are clearly higher than in bare sediments due to the difference in immediate organic supply from plant detritus (Herbert, 1999). Cellular proteins are enzymatically hydrolyzed to their constituent amino acids, which in turn are deaminated to liberate ammonium. Urea is another organic nitrogen compound widely distributed in estuarine and coastal waters, and many indigenous bacteria possess urease that enables to hydrolyze urea and release ammonium to the environment.

Assimilatory nitrate reduction occurs in a wide range of microorganisms, when incorporating nitrogen into biomass. The presence of ammonium represses assimilatory nitrate reduction (Brown, 1988). Most heterotrophic bacteria tend to uptake ammonium and amino acids preferentially if these substrates are available. However, nitrate was the main substrate for planktonic heterotrophic bacteria in the inner part of the Thames estuary due to

high ambient nitrate concentrations (Middelburg and Nieuwenhuize, 2000). This might be a sort of microbial adaptation to their surroundings despite of unfavourable conditions, *i.e.*, preference of nitrogen species utilizations.

As mentioned above, there is a complex relationship among microbial populations, however, the dissimilatory nitrate-reducing bacteria, in charge of the major driving force in the nitrogen flow in estuarine sediments, are to be focused in the following section.

3. Community dynamics of nitrate-reducing bacteria

3.1 The nitrate ammonifying bacteria

Whether the terminal products are solutes or gas, the dissimilatory nitrate-reducing bacteria can be categorized into at least two distinct groups. Nitrate-reducing bacteria originally meant facultative anaerobic bacteria that respire nitrate instead of oxygen. As a result, they produce more reduced species of inorganic nitrogen such as nitrite and nitrogen gas. Since the latter process is specially named as denitrification suggesting a loss of fixed nitrogen to the air (Jeter and Ingraham, 1981), the denitrifying group will be separately mentioned in the next section.

The major microbial agents of dissimilatory nitrate ammonification are found to be a facultatively anaerobic fermentative group, which is currently classified as the gamma-subgroup of Proteobacteria. The ecological significance of this process as a short circuit of nitrogen cycle was proposed by Cole and Brown (1980). A series of microbiological studies based on this viewpoint has supported the existing geochemical data, indicating an

alternative termination of nitrate reduction other than denitrification, simultaneously but independently reported by Sørensen (1978) and Koike and Hattori (1978).

Dunn *et al.* (1980) carried out an enrichment experiment to confirm the possibility of nitrate reduction to ammonium using estuarine sediment slurries with the addition of different carbon sources such as glycerol and acetate. As a result, they found that fermentative bacteria, belonging to *Aeromonas*/*Vibrio* and enterobacteriaceae were most likely to be responsible in the nitrate ammonification process. Then, their group reported that the major end-products of nitrate reduction in a chemostat were nitrite and/or ammonium, along with enzymatic data on nitrate reductase and nitrite reductase. They also observed that the sediments at their sampling site contained high concentration of ammonia throughout the year (Dunn *et al.*, 1978). It has been already known that certain members of enterobacteriaceae produce ammonium via nitrite as a result of *in vitro* nitrate reduction test. However, this physiological pathway had not yet been placed as an ecological counterpart of nitrogen cycle in nature.

Predominant nitrate-reducing bacteria in the River Tay estuary were identified as the *Aeromonas*/*Vibrio* group, and they tended to excrete ammonia under anaerobic and nitrogen limiting conditions (Macfarlane and Herbert, 1982). Few field data are available from estuaries, however, diverse denitrifying bacteria migrated from rivers, soils and sewage sludge could be deposited in the downstream estuarine sediments. Fermentative nitrate-reducing bacteria were more diverse and in greater numbers than

respiratory denitrifying bacteria in soil environments due to the presence of typical soil inhabitants, *Streptmyces* and *Bacillus* spp. (Shirey and Sexstone, 1989). A spore-forming obligately anaerobic *Clostridium perfringens*, possibly derived from sewage works, also could form ammonium (Hasan and Hall, 1975) from nitrate after having settled in the sediment. Another anaerobic *Desulfovibrio desulfuricans* can utilise nitrate as well as sulphate as a terminal electron acceptor and the growth yields was enhanced by the presence of nitrate in a microcosm (Keith and Herbert, 1983).

3.2 The nitrate denitrifying bacteria

Denitrification is, in a narrow sense, defined as the reduction of nitrate to a gaseous product. Actually, however, this concept of denitrification has been expanded. For example, some denitrirying strains produce soluble nitrous oxide gas instead of visible nitrogen gas bubbles; hence gas production from nitrate is not always the reliable criteria of the presence of denitrifying bacteria (Patriquin and Knowles, 1974). In addition, certain genera such as *Alcaligenes* and *Pseudomonas* are incapable of utilising nitrate but not nitrite, which is called "nitrite-dependent" denitrification (Vangnai and Klein, 1974). Furthermore, Pichinoty *et al.* (1979) isolated *Bacillus* sp. that was able to utilise nitric oxide as a terminal electron acceptor during denitrification.

The taxonomic distribution of denitrifying bacteria in nature is very wide. However, the heterotrophic *Pseudomonas* and *Alcaligenes* genera are most frequently isolated from fertilized soils and sewage sludge. A few genera, a lithotrophic *Thiobacillus denitrificans* and a phototrophic *Rhodopseudomonas* (now

amended as the genus *Rhodobacter*) variant, are metabolically versatile but capable of denitrificaion in a particular niche. These genera are in the alpha- and beta-sub groups of proteobacteria.

However, there are likely to be a few exceptions to the differentiation of denitrifying and amminifying groups of bacteria, overlapping each other. For example, *Pseudomonas putrefaciens* has the capacity to dissimilate nitrate to ammonium as well as to nitrous oxide (Samuelson, 1985). *Citrobacter* sp. isolated from a soil could reduce nitrite to ammonium and nitrous oxide under a certain condition (Smith, 1982).

Semi-quantitative field surveys indicated that *Pseudomonas* and *Alcaligenes* were the most abundant genera in a polluted river (Nakajima, 1982), a eutrophic lake (Terai, 1979) coastal water (Sugahara *et al.*, 1986), and various soils (Gamble *et al.*, 1977). Novel species of denitrifying isolates have been found from some man-made habitats, particularly in wastewater treatment modules. A photosynthetic denitrifier, *Rhodopseudomonas* (*Rhodobacter*) *spaeroides* forma sp. *denitrificans*, for example, was originally isolated from a waste stabilization lagoon of food industry in Japan (Satoh *et al.*, 1976). In contrast, artificial *Thiospaera panthtropha* biofilms have been applied to a wastewater treatment module adopting the combined simultaneous nitrification-denitrificaion process in Delft (Dalsgaard *et al.*, 1995). This means that the specific denitrifying niches can be found in wastewater treatment and estuarine systems, both having the similar conditions such as higher nitrogen content and a continuous flow system.

3.3. Selective factors of nitrate-reducing bacteria

Since nitrate ammonifying and denitrifying bacteria share their functional niche, further attention should be focused on their interactions, namely, a commensalism and a competition in a mixed culture. In other words, what kind of environmental variables would determine the population dynamics of these two groups and the resulting partitioning of nitrate between two fates in estuarine sediments?

First of all, the results of enrichment experiments by Dunn *et al.* (1980) indicated that organic substrate, salinity and oxygen status were some of the possible factors. They found that the presence of nitrate under anaerobic condition remarkably induced the dissimilatory nitrate reductase, though salinity did little to affect the sediment community in the Kingoodie estuary, Scotland (Dunn *et al.*, 1978).

Herbert (1982) pointed out that nitrate reduction was energetically superior to proper fermentation, and the increase of the density of nitrate-reducing fermentative bacteria was reasonable. This explains the competition between two types of fermentative bacteria. Although nitrite produced by nitrate-reducing bacteria would be beneficial to nitrite dependent species as a commensalism (Lloyd, 1999), there must be a competition for nitrate/nitrite as the common substrate between ammonifying and denitrifying bacteria in natural environments.

Tiedje *et al.* (1982) suggested that the available electron donor/acceptor ratios affected the overall distribution of denitrifying populations in the environments. Based on the calculation of theoretical energy yield,

denitrification is favoured when carbon source is limiting, while ammonification is favoured when electron acceptor is limiting.

King and Nedwell (1985) demonstrated using sediment slurries that denitrification dominated at high nitrate concentrations, while ammonification dominated at low nitrate concentrations. Rehr and Klemme (1989) also found the same conclusion that the C/N raio needed for denitrification is far lower than that for ammonification, employing chemostats of a pure culture basis.

In natural environments, temperature would be the most primitive selective pressure against mixed populations. Based on this idea, Ogilvie *et al.* (1997a) carried out chemostat experiment under different temperatures. They proposed that denitrifying bacteria predominate in estuarine sediments at low temperature, whereas fermentative ammonifying bacteria become better competitors for nitrate at higher temperature. Thus, in the temperate regions, seasonal changes in ambient temperature result in selection of two types of nitrate-reducing bacteria in estuarine sediments.

Lloyd (1999) showed the evidence of a switching of metabolism by measuring the end-products of nitrate reduction using pure cultures. One of the regulating factors was a C/N ratio, and the high C/N ratio stimulated proper fermentation rather than nitrate ammonification when glucose was fed to *Klebsiella pneumoniae* and *Serratia liquefaciens*. Another factor was an incubation temperature, and low temperature shifted those bacteria from ammonium formation to nitrous oxide formation. When those bacteria were mixed with *Alcaligenes faecalis*, *K. pneumoniae* and *S. liquefaciens* accumulated ammonium at low temperature, while nitrite dependent *A. faecalis* produced

nitrogen gas. These results partially suggest that *in situ* metabolic pathway of nitrate reduction is rather complicated in terms of both biotic and abiotic conditions such as environmental temperatures and species interactions.

Enrichment and maintenance of benthic bacterial communities are ensured by constant loadings of nitrate from a sewage outfall in the estuarine system. In such a particular situation, the nitrate-reducing bacteria exhibited faster rates of nitrate reduction than usual. Moreover, the proportion of denitrification, rather than ammonification, also increased with the ambient nitrate concentrations as shown in the Colne estuary (King and Nedwell, 1987). Natural bacterial communities are likely to install a self-regulation mechanism themselves in response to the environmental stress. A halophilic fermentative bacterium reported as an agent capable of both denitrification and nitrate ammonification in a single strain basis (Shieh and Liu, 1996).

4. Molecular aspects of dissimilatory nitrate reduction

4.1 Perspectives of non-cultural approach

It is often said that bacteria in nature are quite different from those *in vitro*. Since the establishment of the acridine orange epifluorescence direct count (AODC) by Hobbie *et al.* (1977), it has long been known that there is an enormous gap between direct counts and viable counts by several orders of magnitude when enumerating surviving bacteria. The direct viable count (DVC) by counting elongated cells caused by the inhibition of cell division would be one solution (Kogure *et al.*, 1979). Hence, the difference between an AODC and a DVC could be realistic estimation of native aquatic bacteria being

in the viable but nonculturable (VBNC) status (Roszak and Colwell, 1987).

The traditional isolation-identification approach of a viable count basis may be focused but biased between reconstructed features and the actual microbial community structure in the environment. To analyse the structure in sediments, chemical analysis of whole biomass using cellular fatty acids (Parkes, 1987) and isoprenoid quinones (Hiraishi and Kato, 1999) as biomarkers could be used. However, these results can reflect chemotaxonomical chracteristics but not always present phylogenetic and functional information on the original community. Alternatively, analysis of the PCR products of 16S rDNAs, and more specifically, functional genes coding for specific pathways would be more useful for these particular purposes.

A simple and rapid method of extracting DNA from soil or sediment samples has been previously published (Zhou *et al.*, 1996). In addition to DNA extraction, analytical procedures such as restriction fragment length polymorphism (RFLP) analysis to evaluate the diversity of genomic (Ward, 1995) and environmental DNA (Moyer *et al.*, 1994) has been developed. Moreover, terminal restriction fragment length polymorphism (T-RFLP) using a labeled fluorescent dye and an automated capillary analyser is a useful tool for assessing the diversity of bacterial assemblage rapidly, comparing their community structure with a high reproducibility (Osborn *et al.*, 2000)

4.2 Detection of functional genes as their presence and expression

There must be still a gap between the presence of the target functional gene and the activity of the target enzyme, similar to the difference between total

counts and viable counts. Since mRNA has a short lifetime and is produced immediately prior to protein synthesis, detection of mRNA using a reverse transcriptase PCR (RT-PCR) gives an estimation of real expression of enzymatic potential (Pepper, 1997).

The RT-PCR has a wide range of possible applications in ecological studies of microbial community. Wawer *et al*. (1997) and Zani *et al.* (2000) successfully adopted the RT-PCR technique for detecting the actively expressing functional genes that sulphate-reducing and nitrogen-fixing bacteria have, respectively. Later, the diversity of expressed denitrification genes was investigated in the Colne estuary (Nogales, *et al*., 2002), exhibiting differences in geographical distribution of the relevant functional genes along the estuarine system in accordance with the gradient of nitrate loading to the overlying water.

However, whether DNA or RNA indicated as presence or expression of genes, most of the molecular biological studies on natural microbial communities has initially been focused on phylogenetic 16S rDNA, then specific functional genes involved in the biogeochemical cycles such as dinitrogenase (Kirshtein *et al*., 1991), ammonium monooxygenase (Voytek and Ward, 1995), methane monooxygenase (Henckel *et al*., 1999), dissimilatory sulfite reductase (Minz *et al*., 1999), nitric oxide reductase (Braker and Tiedje, 2003), and dissimilatory nitrite reductase (Mohan *et al*., 2004), encoding nitrite reduction to ammonia from wastewater treatment modules. However, none of the molecular approach towards nitrate ammonification in estuarine sediments has been so far tried.

4.3 Genes coding for nitrate reduction

Each of the microbial metabolic pathways is catalysed by the specific enzyme. Besides, each enzyme is encoded by the specific functional gene. A series of the nitrate reduction processes and each gene that involved in each step are shown in **Figure 1-2**.

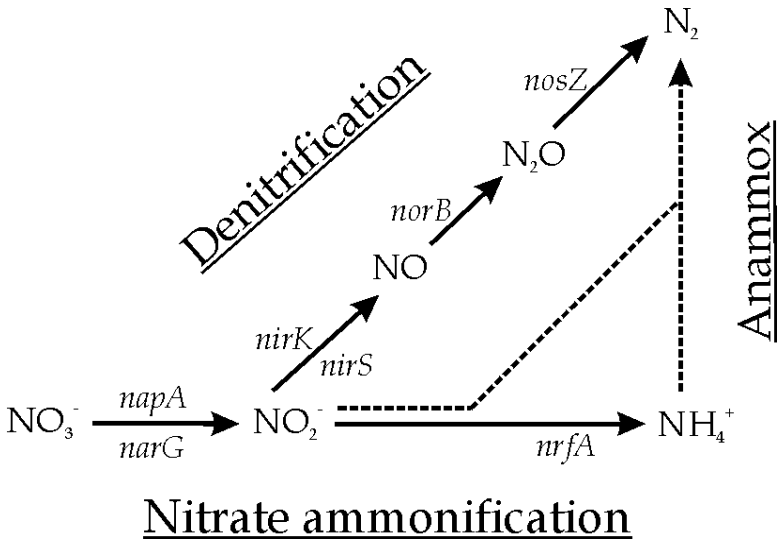


Figure 1-2 Dissimilatory nitrate reduction and the relevant functional genes

Recently, diverse bacterial respirations and their regulating genes have been studied for the sake of physiological and evolutionary interests. The respiratory flexibility among the *Bacteria* and *Archaea* exceeds any other microorganisms. Thus, their versatile functions would contribute to colonize ancient and modern extreme environments on the Earth (Richardson, 2000).

Since research on respiratory enzymes contributes to clarify the survival mechanism of opportunistic pathogens outside the host organisms, this approach would also be useful for microorganisms of environmental origin. As mentioned in the previous section, the activity of any enzyme would be

influenced by environmental variables such as temperatures and substrates. Therefore, molecular analysis on the specific functional genes gives reliable results. In the case of functional genes encoding the nitrate reduction process, the detection of each gene in genomic DNA and community DNA contributes to the fundamental information on ecological research. Recent information on each functional gene for the nitrate reduction process and its developmental state of PCR protocol is as follows.

Nitrite reductases are unique to denitrifying bacteria but not to ammonifying bacteria. They are distributed in the periplasm, and divided into two types, namely, a copper-containing *nirK* and a heme-containig *nirS* (Smith and Tiedje, 1992). Every denitrifying bacterium has exclusively one of each. Generally, the most abundant denitrifiers such as *Pseudomonas* and *Alcaligenes* have the former, while *Ochrobacterum* and *Rhodobacter* have the latter.

Primer pairs for *nirS* and *nirK* and the PCR protocol using a "touchdown" mode has been published elsewhere (Braker *et al.*, 1998). They applied this PCR amplification technique as a molecular marker to the community DNAs extracted from marine sediments, and reported that *nirS* was more frequently detected than *nirK* (Braker *et al.*, 2000). Then, they compared the diversity of community structure by using a RFLP analysis. Their results indicated that a higher diversity was found in the *nirS* clones rather than the *nirK* clones. They therefore pointed out that there must be so far unknown and/or unculturable denitrifiers in environmental samples. Interestingly, the denitrifying *nirK* gene was found in some of autotrophic ammonia-oxidizing bacteria (Casciotti and Ward, 2001).

There are two distinct types of nitrate reductases, namely, a membrane-bound *Nar* and periplasmic *Nap*. *Nar* is expressed under the conditions of low oxygen tension and high nitrate concentration, while *Nap* is usually expressed under the conditions of high oxygen tension and low nitrate concentration. Thus, *Nar* is a high-substrate-induced operon (Wang *et al.*, 1999). However, the expression of *Nap* is not induced by nitrate and anaerobic condition. *Nap* is thought to be more important for the growth, because certain human pathogens encode only the *Nap* enzyme (Richardson, 2000). Both ammonifying and denitrifying bacteria are considered to have these *Nar* and/or *Nap* genes, except for some nitrite dependent isolates.

Primer pairs and its PCR protocol for amplifying the *narG* gene have been already published (Gregory *et al.*, 2000). By using the nested PCR amplification, PCR products around 1,600 bp after the 1st round and 500 bp after the 2nd round were obtained when employing genomic and community DNAs. According to their result, *narG* sequences amplified from bacterial and sediment DNAs from a freshwater pond were rather homogenous. Primers for amplifing the *napA* gene have been developed by the same group (Flanagan *et al.*, 1999). As a PCR procedure, the nested PCR was employed for amplifying *napA*. Since reduction of nitrate in the periplasm is not sensitive to oxygen, *Nap* enables the bacteria having *Nap* to grow under aerobic condition. Thus, *Nap* may make a contribution to the flow of nitrate/nitrite in the environments (Carter *et al.*, 1995). However, ecological significance of these two competitive nitrate reductases is still unclear and to be clarified.

Nitrous oxide reductase is a periplasmic copper-containing *Nos* enzyme that

converts nitrous oxide to nitrogen gas. This enzyme is sensitive to oxygen and inhibited by low pH. Scala and Kerkhof (1998) developed the primer pairs and the PCR protocol to investigate the phylogenetic relationship of denitrifying bacterial communities in the sediments (Scala and Kerkhof, 1999).

Diversity of the *nrfA* gene, encoding nitrite reduction to ammonia, has been recently investigated in anaerobic wastewater treatment reactors (Mohan *et al*., 2004), suggesting its widespread taxonomical distribution and possible competition with denitrification for nitrite in the environment.

These amplified PCR products of each functional gene could be sequenced and examined in terms of diversity so as to compare among sampling seasons, sites and habitats, leading to an ecological study on *in situ* bacterial community structure with culture-independent procedure.

5. Purpose of this study

The following aims were addressed in this study focused on the nitrate-reducing bacteria in the hypernutrified habitats, particularly involved in the nitrate ammonification process:

1) Screening nitrate-reducing bacteria from the environment,
2) Enrichment of nitrate-reducing bacteria using activated sludge,
3) Developing and optimizing the PCR protocols for the *nrfA* gene,
4) Amplifying and sequencing the *nrfA* gene from the sediment,
5) Analysing distribution patterns using phylogenetic properties.

References

An S, Joye SB (2001) Enhancement of coupled nitrification-denitrification by benthic photosynthesis in shallow estuarine sediments, *Limnol Oceanogr*, **46**: 62-74

Aziz SAA, Nedwell DB (1979) Microbial nitrogen transformation in the salt marsh environment, *Ecological Progress in Coastal Environments* (Ed by Jefferies RL and Davy AJ), Blackwell Scientific Publications, Oxford, 385-398

Braker G, Fesefeldt A, Witzel K-P (1998) Development of PCR primer systems for amplification of nitrite reductase gene (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples, *Appl Environ Microbiol*, **64**: 3769-3775

Braker G, Zhou J, Wu L, Devol AH, Tiedje JM (2000) Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in Pacific northwest marine sediment communities, *Appl Environ Microbiol*, **66**: 2096-2104

Braker G, Tiedje JM (2003) Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples, *Appl Environ Microbiol*, **69**: 3476-3483.

Brown CM (1988) Nitrate metablism by aquatic bacteria, *Methods in Aquatic Bacteriology*(Ed by Austin B), John Wiley & Sons, Chichester, 367-388

Capone DG, Kiene RP (1988) Comparison of microbial dynamics in marine and freshwater sediments: contrasts in anaerobic carbon catabolism, *Limnol*

Oceangr, **33**: 725-749

Carter JP, Hsiao YH, Spiro S, Richardson DJ (1995) Soil and sediment bacteria capable of aerobic nitrate respiration, *Appl Environ Microbiol*, **61**: 2852-2858

Casciotti KL, Ward BB (2001) Dissimilatory nitrite reductase genes from autotrophic ammonia-oxidizing bacteria, *Appl Environ Microbiol*, **67**: 2213-2221

Cole JA, Brown CM (1980) Nitrite reduction to ammonia by fermentative bacteria: a short circuit in the biological nitrogen cycle, *FEMS Microbiol Lett*, **7**: 65-72

Dalsgaard T, de Zwart J, Robertson LA, Kuenen JG, Revsbecj NP (1995) Nitrification, denitrification and growth in artificial *Thiosphaera pantotropha* biofilms as measured with a combined mirosensor for oxygen and nitrous oxide, *FEMS Microbiol Ecol*, **17**: 137-148

Dong LF, Nedwell DB, Underwood GJC, Thornton DCO, Rusmana I (2002) Nitrous oxide formation in the Colne estuary, England: the central role of nitrite, *Appl Environ Microbiol*, **68**: 1240-1249

Dong LF, Thornton DCO, Nedwell DB, Underwood GJC (2000) Denitrification in sediments of the River Colne estuary, England, *Mar Ecol Prog Ser*, **203**: 109-122

Dunn GM, Herbert RA, Brown CM (1978) Physiology of denitrying bacteria from tidal mudflats in the River Tay, Physiology and behaviour of marine organisms (Ed by McLusky DS and Berry AJ), Pergamon Press, Oxford, 135-140

Dunn GM, Wardell JN, Herbart RA, Brown CM (1980) Enrichment, enumeration and characterisation of nitrate-reducing bacteria present in sediments of the River Tay estuary, *Pro Royal Soc Edinburgh*, **78B**: s47-s56

Flanagan DA, Gregory LG, Carter JP, Karakas-Sen A, Richardson DJ, Spiro S (1999) Detection of genes for periplasmic nitrate reductase in nitrate respiring bacteria and in community DNA, *FEMS Microbiol Lett*, **177**: 263-270

Gamble TN, Betlach MR, Tiedje JM (1977) Numerically dominant denitrifying bacteria from world soils, *Appl Environmen Microbiol*, **33**: 926-939

Gregory LG, Karakas-Sen A, Richardson DJ, Spiro S (2000) Detection of genes for membrine-bound nitrite reductase in nitrate-respiring bacteria and in community DNA, *FEMS Microbiol Lett*, **183**: 275-279

Hasan SM, Hall JB (1975) The physiological function of nitrate reduction in *Clostridium perfringens*, *Jour Gen Microbiol*, **87**: 120-128

Henckel T, Friedrich M, Conrad R (1999) Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase, *Appl Envir Microbiol*, **65**: 1980-1990.

Herbert RA (1982) Nitrate dissimilation in marine and estuarine sediments, *Sediment Microbiology* (Ed by Nedwell DB and Brown CM), Academic Press, London, 53-71

Herbert RA (1999) Nitrogen cycling in coastal marine ecosystems, *FEMS Microbiol Rev*, **23**: 563-590

Hiraishi A, Kato K (1999) Quinone profiles in lake sediments: implications for microbial diversity and community structure, *J Gen Appl Microbiol*, **45**: 221-227

Hobbie JE, Daley RJ, Jasper S (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy, *Appl Environ Microbiol*, **33**: 1225-1228

Horan NJ (1990) *Biological Watewater Treatment Systems-Theory and Operation*, John Wiley & Sons, Chichester, 310pp

Jeter RM, Ingraham JL (1981) The denitrifying prokaryotes, *The Prokaryotes-A Handbook on Habitats, Isolation, and Identification of Bacteria Vol 1* (Ed by Starr MP, Stolp H, Truper HG, Balows A, Schlegel HG), Springer Verlag, Berlin, 913-925

Jones JG, Downes MT, Talling IB (1980) The effect of sewage effluent on denitrification in Grasmere (English Lake District), *Freshwater Biol*, **10**: 341-359

Keith SM, Herbert RA (1983) Dissimilatory nitrate reduction by a strain of *Desulfovibrio desulfuricans*, *FEMS Microbiol Lett*, **18**: 55-59

King D, Nedwell DB (1985) The influence of nitrate concentration upon the end-productss of nitrate dissimilation by bacteria in anaerobic salt marsh sediment, *FEMS Microbiol Ecol*, **31**: 23-28

King D, Newdwell DB (1987) The adaptation of nitrate-reducing bacterial communities in estuarine sediments in response to overlying nitrate load, *FEMS Microbiol Ecol*, **45**: 15-20

Kirshtein JD, Paerl HW, Zehr J (1991) Amplification, cloning, and sequencing of

a *nifH* segment from aquatic microorganisms and natural communities, *Appl Environ Microbiol*, **57**: 2645-2650

Kogure K, Simidu U, Taga N (1979) A tentative direct microscopic method for counting living marine bacteria, *Can J Microbiol*, **25**: 415-420

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

Lloyd, DL (1999) Temperature effects on competition, selection and physiology of estuarine nitrate-respiring bacteria, PhD thesis, Univ of Essex, 298pp

Macfarlane GT, Herbert RA (1982) Nitrate dissimilation by *Vibrio* spp. Isolated from estuarine sediments, *Jour Gen Microbiol*, **128**: 2463-2468

Malone TC (1984) Anthropogenic nitrogen loading and assimilation capacity of the Hudson River estuarine system, USA, *The Estuary as a Filter* (Ed by Kennedy VS), Academic Press, Orlando, 291-311

McCready RGL, Gould WD, Cook FD (1983) Respiratory nitrate reduction by *Desulfovibrio* sp., *Arch Microbiol*, **135**: 182-185

Middelburg JJ, Nieuwenhuize J (2000) Nitrogen uptake by heterotrophic bacteria and phytoplankton in the nitrate-rich Thames estuary, *Mar Ecol Prog Ser*, **203**: 13-21

Minz D, Flax JL, Green SJ, Muyzer G, Cohen Y, Wagner M, Rittmann BE, and Stahl DA (1999) Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterized by comparative analysis of dissimilatory sulfite reductase genes, Appl Envir Microbiol, 65: 4666-4671

Mohan SB, Schmid M, Jetten M, Cole J (2004) Detection and widespread

distribution of the *nrfA* gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification, *FEMS Microbiol Ecol*, **49**: 433-443

Moyer CL, Dobbs CF, Karl DM (1994) Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat community at an active, hydrothermal vent system, Loihi Seamount, Hawaii, Appl Environ Microbiol, **60**:871-879

Mulder A, van de Graaf AA, Robertson LA, Kuenen JG (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor, *FEMS Microbiol Ecol*, **16**: 177-184

Nakajima T (1982) Distribution of denitrifying bacteria and its controlling factors in freshwater environments, *Jpn J Limnol*, **43**: 17-26

Nedwell DB (1975) Inorganic nitrogen metabolism in a eutrophicated tropical mangrove estuary, *Water Res*, **9**: 221-231

Nedwell DB, Jickells TD, Trimmer M, Sanders R (1999) Nutrients in estuaries, *Adv in Ecol Res*, **29**: 43-92

Nogales B, Timmis KN, Nedwell DB, Osborn AM (2002) Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA, *Appl Envir Microbiol*, **68**: 5017-5025

Ogilvie BG, Rutter M, Nedwell DB (1997a) Selection by temperature of nitrate-reducing bacteria from estuarine sediments: species composition and competition for nitrate, *FEMS Microbiol Ecol*, **23**: 11-22

Ogilvie B, Nedwell DB, Harrison RM, Robinson A, Sage A (1997b) High nitrate, muddy estuaries as nitrogen sinks: the nitrogen budget of the River Colne estuary (United Kingdom), *Mar Ecol Prog Ser*, **150**: 217-228

Osborn AM, Moore ERB, Timmis KN (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics, *Environ Microbiol*, **2**: 39-50

Owens, NJP (1986) Estuarine nitrification: a natually occurring fluidized bed reaction? *Estuar Coastal Shelf Sci*, **22**: 31-44

Parkes RJ (1987) Analysis of microbial communities within sediments using biomakers, *Ecology of Microbial Communities* (Ed by Fletcher M, Gray TRG, Jones JG), Cambridge Univ Press, Cambridge, 147-177

Patriquin DG, Knowles R (1974) Denitrifying bacteria in some shallow-water marine sediments: enumeration and gas production, *Can J Microbiol*, **20**: 1037-1041

Pepper, IL (1997) PCR: applications for plant and soil microbes, *Manual of Environmental Microbiology* (Ed by Hurst SJ), ASM Press, Washington DC, 437-444

Pichinoty, F, Mandel M, Garcia J-L (1979) The properties of novel mesophilic denitrifying Bacillus cultures found in tropical soils, *Jour Gen Microbiol*, **115**: 419-430

Rehr B, Klemme J-H (1989) Competition for nitrate between denitrifying *Pseudomonas stutzeri* and nitrate ammonifying enterobacteria, *FEMS Microbial Ecol*, **62**: 51-58

Richardson DJ (2000) Bacterial respiration: a flexible process for a changing environment, *Microbiology*, **146**: 551-571

Robertson LA, Kuenen JG (1983) *Thiospaera pantptropha* gen. nov. sp., a facultatively anaerobic, facultatively autotrophic sulphur bacterium, *Jour Gen Microbiol*, **129**: 2847-2855

Robertson LA, Kuenen JG (1992) Nitrogen removal from water and waste, *Microbial Control of Pollution* (Ed by Fry JC, Gadd GM, Herbert RA, Jones CW, Watson-Craik IA), Cambridge Univ Press, Cambridge, 227-267

Roszak DB, Colwell RR (1987) Survival strategies of bacteria in the natural environemt, *Microbiol Rev*, **51**: 365-379

Samuelson, M-O (1985) Dissimilatory nitrate reduction to nitrite, nitrous oxide, and ammonium by *Pseudomonas putrefaciens*, *Appl Environ Microbiol*, **50**: 812-815

Satoh S, Hoshino Y, Kitamura H (1976) *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*, a denitrifying strain as a subspecies of *Rhodopseudomonas spaeroides*, Arch Microbiol, **108**: 265-269

Scala DJ, Kerkhof LJ (1998) Nitrous oxide reductase (*nosZ*) gene-specific primers for detection of denitrifiers and three *nosZ* genes from marine sediments, *FEMS Microbiol Lett*, **162**: 61-68

Scala DJ, Kerkhof LJ (1999) Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments, *Appl Environ Microbiol*, **65**: 1681-1687

Seitzinger, SP (1988) Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance, *Limnol Oceanogr*,

33: 702-724

Shieh WY, Liu CM (1996) Denitrification by a novel halophilic fermentative bacterium, *Can J Microbiol*., **43**: 507-514.

Shirey JJ, Sexstone AJ (1989) Denitrification and nitrate-reducing bacterial populations in abandoned and reclaimed minesoils, *FEMS Microbiol Ecol*, **62**: 59-70

Smith GB, Tiedje JM (1992) Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria, *Appl Environ Microbiol*, **58**: 376-384

Smith MS (1982) Dissimilatory reduction of NO_2^- to NH_4^+ and N_2O by a soil *Citrobacter* sp., *Appl Environ Microbiol*, **43**: 854-860

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

Sugahara I, Hayashi K, Kimura T (1986) Distribution and generic composition of denitrifying bacteria in coastal and oceanic waters, *Bull Jpn Soc Sic Fish*, **52**: 497-503

Terai H (1979) Taxonomic study and distribution of denitrifying bacteria in Lake Kizaki, *Jpn J Limnol*, **40**: 81-92

Tiedje JM, Sexstone AJ, Mayrold DD, Robinson JA (1982) Denitrification: ecological niches, competition and survival, *Antonie van Leeuwenhoek*, **48**: 569-583.

Trimmer M, Nedwell DB, Sivyer DB, Malcolm SJ (2000) Seasonal benthic organic matter mineralisation measured by oxygen uptake and

denitrification along a transect of the inner and outer River Thames estuary, UK, *Mar Ecol Prog Ser*, **197**: 103-119

Vangnai S, Klein DA (1974) A study of nirtite-dependent dissimilatory micro-organisms isolated from Oregon soils, *Soil Biol Biochem*, **6**: 335-339

Voytek MA, Ward BB (1995) Detection of ammonium-oxidizing bacteria of the beta-subclass of the class Proteobacteria in aquatic samples with the PCR, *Appl Envir Microbiol*, **61**: 1444-1450

Wang H, Tseng C-P, Gunsalus RP (1999) The *napF* and *narG* nitrate reductase operons in *Escherichia coli* are differentially expressed in response to submicromolar concentrations of nitrate but not nitrite, *Jour Bacteriol*, **181**: 5303-5308

Ward BB (1995) Diversity of culturable denitrifying bacteria: limits of rDNA RFLP analysis and probes for the functional gene, nitrite reductase. *Arch Microbiol*, **163**: 167-175

Wawer C, Jetten MSM, Muyzer G (1997) Genetic diversity and expression of the [NiFe] hydrogenase large-subunit gene of *Desulfovibrio* spp. in environmental samples, *Appl Environ Microbiol*, **63**: 4360-4369

Zani S, Mellon MT, Collier JL, Zehr JP (2000) Expression of *nifH* genes in natural microbial assemblages in Lake George, New York, detected by reverse transcriptase PCR, *Appl Environ Microbiol*, **66**: 3119-3124

Zhou J, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition, *Appl Environ Microbiol*, **62**: 316-322

Chapter 2

Nitrate-reducing bacteria isolated from various kinds of waters

Abstract

Nitrate is of significance in aquatic environments, being a possible substrate both for denitrification and other dissimilatory reduction from nitrate/nitrite. In this chapter, a main attention has been focused on the end-products of nitrate/nitrite dissimilation by heterotrophic bacterial isolates from various kinds of water, ranging from river water to sewage related samples. More than 50% of the isolates accumulated nitrite (NO_2^-) from nitrate (NO_3^-), *i.e.* nitrate-reducing bacteria predominated in raw sewage and polluted river water samples. Some (4-26%) of the isolates produced dinitrogen gas (N_2) from nitrate, *i.e.* denitrifying bacteria predominated in settling sewage which was contaminated with activated sludge. Few (2-4%) of the isolates produced N_2 gas from nitrite, but not from nitrate, *i.e.* the nitrite-dependent denitrifiers. About five times' larger numbers of denitrifying bacteria were detected by using nitrate broth than by using Giltay broth. This result is considered to be evidence that the nitrate broth supports the growth of a wide variety of denitrifying species of bacteria. Most of the denitrifying isolates belonged to the genera *Pseudomonas* and *Alcaligenes*. The ammonifying groups of bacteria were the following fermentators: *Aeromonas*, *Klebsiella*, *Escherichia* and a *Vibrio*-like bacterium. A few of the *Aeromonas* isolates could produce gas both from the nitrate broth and the OF (oxidation-fermentation) test agar.

1. Introduction

Nitrogen as an inert gas is abundantly reserved in the atmosphere. Fixed nitrogen in soils and water can be returned to the air via denitrification, thereby making the biological nitrogen cycle. There are various metabolic pathways of dissimilatory nitrate reduction as shown in **Figure 2-1**. This suggests a close interaction between bacterial community and the environment, in which the conditions can affect the bacteria community dynamics *in situ*, whilst the populations can form the environment by producing their own metabolites.

Nitrate in the hypernutrified waters discharged from sewage effluent outfalls is expected to be eventually removed from the water by denitrification. However, nitrate can be returned to ammonia by nitrate ammonification (or dissimilatory nitrate reduction to ammonia, DNRA).

Nitrate ammonification was first reported from costal marine sediments by Koike and Hattori (1978) and Sørensen (1978) independently, suggesting that the final end-products may be determined by the biogeochemical features of sediments. This implies that the necessity of analysis of bacterial community structure which could affect the major metabolic pathways of their habitats.

Cole and Brown (1980) reported that certain members of fermentative anaerobic bacteria could reduce nitrate to ammonia via nitrite. Thus, the investigation on metabolic patterns of the nitrate-reducing bacterial community may predict the fate of nitrate in the environment. Based on this viewpoint, nitrate-reducing bacteria were isolated from various kinds of waters and the metabolic patterns were examined in this study, though this culture-depending approach has methodological limitations.

2. Materials and Methods

2.1 Sampling procedure

All the water samples were collected in a sterile glass bottles and inoculated onto agar plates. Raw sewage, settled sewage and activated sludge samples were collected from the Shingashi Treatment Plant, Tokyo, Japan. River water samples were collected from sites such as the Hamura (>2 mg/l BOD) and Chofu (>3 mg/l BOD) Bridges of the Tamagawa River in Tokyo and the Ippei (26 mg/l BOD) Bridge of the Sakagawa River, Chiba, Japan. The water samples discharged from the Shingashi Treatment Plant were also sampled from the Shigashi River at the Hayase (upstream) and Tokumaru (downstream) Bridges (15 mg/l BOD). In addition, the polished sewage effluent (5 mg/l BOD) was collected from an artificial creek from the Shingashi Treatment Plant.

2.2 Bacterial counts

Aerobic heterotrophic bacteria were enumerated on Sakurai's agar plate (Tokyo Bureau of Sewerage, 1990) using spread plate technique. The plates inoculated with river water were incubated at 20°C for 14 days, whilst those with other sewage-related samples were incubated at 28°C for 14 days.

Preliminary examination on the enumeration and isolation were investigated using both media, Giltay and nitrate broth (Tokyo Bureau of Sewerage, 1990). Time course of the nitrogen gas production in Durham tubes were observed in this preliminary examination at 28°C for 20 days.

2.3 Bacterial isolation

Fifty colonies were isolated and purified from each plate after enumeration. These stock cultures were maintained using the Sakurai's slant without glucose.

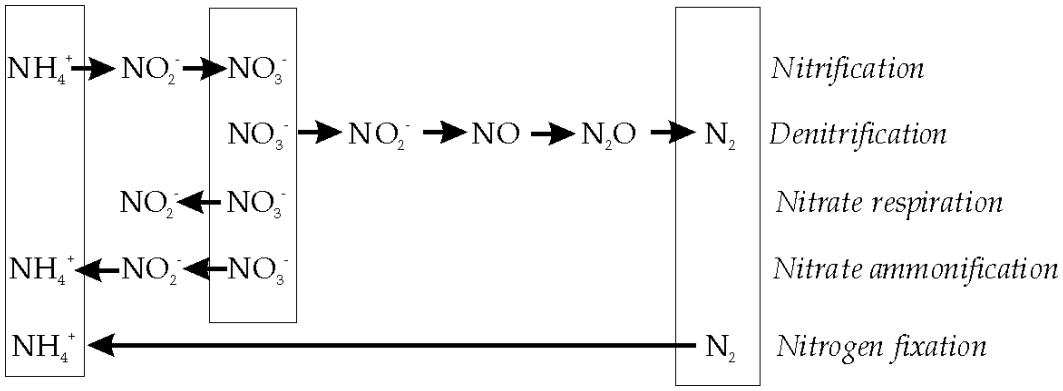


Figure 2-1 Metabolism of inorganic nitrogen

Table 2-1 Cultural media for denitrifying bacteria

Ingredients	Giltay broth	Nitrate broth
Polypeptone		5.0
Yeast extract		1.0
KH_2PO_4	1.0	0.1
KNO_3	1.0	1.0
Asparagine	1.0	
Sodium citrate	8.5	
$MgSO_4$ $7H_2O$	1.0	
$FeCl_3$ $6H_2O$	0.05	
$CaCl_2$ $2H_2O$	0.2	

(g/l, pH adjusted to 7.1)

2.4 Differentiation of metabolic patterns

Each isolate were inoculated into both Giltay and nitrate broth with Durham tubes, as shown in **Table 2-1**. The production of gas in the tubes was monitored. The presence of gas indicates denitrification. Negative tubes were examined by adding a small amount of Griess-Romijn's reagent (Tokyo Bureau of Sewerage, 1990). The tubes showing red colour indicate the presence of nitrite. The negative tubes were examined again by adding a small amount of zinc powder. The tubes showing red colour indicate the nitrate remained, whilst the tubes without colour change were implied the possibility of producing nitrous oxide or ammonia without gas production. These isolates without gas production were inoculated into semi-solid agar tubes of Hugh-Leifson's medium (Nissui Pharmacy, Tokyo). The tubes showing yellow colour, sometimes with gas production, indicate the formation of acid from glucose. In combination with these test results, two types of metabolic patterns, *i.e.* non-fermentators for nitrous oxide-accumulating and fermentators for ammonia-accumulating isolates were tentatively differentiated.

In addition, nitrite reduction was also examined in the nitrite broth with replaced nitrate by nitrite, as a nitrogen content of 600mg/l. This enabled identification of nitrite-dependent isolates that may lack the enzyme capable of catalyzing nitrite reduction but not nitrate reduction (Vangnai and Klein, 1974). A tentative scheme for differentiating the nitrate/nitrite reduction patterns is summarized in **Figure 2-2**.

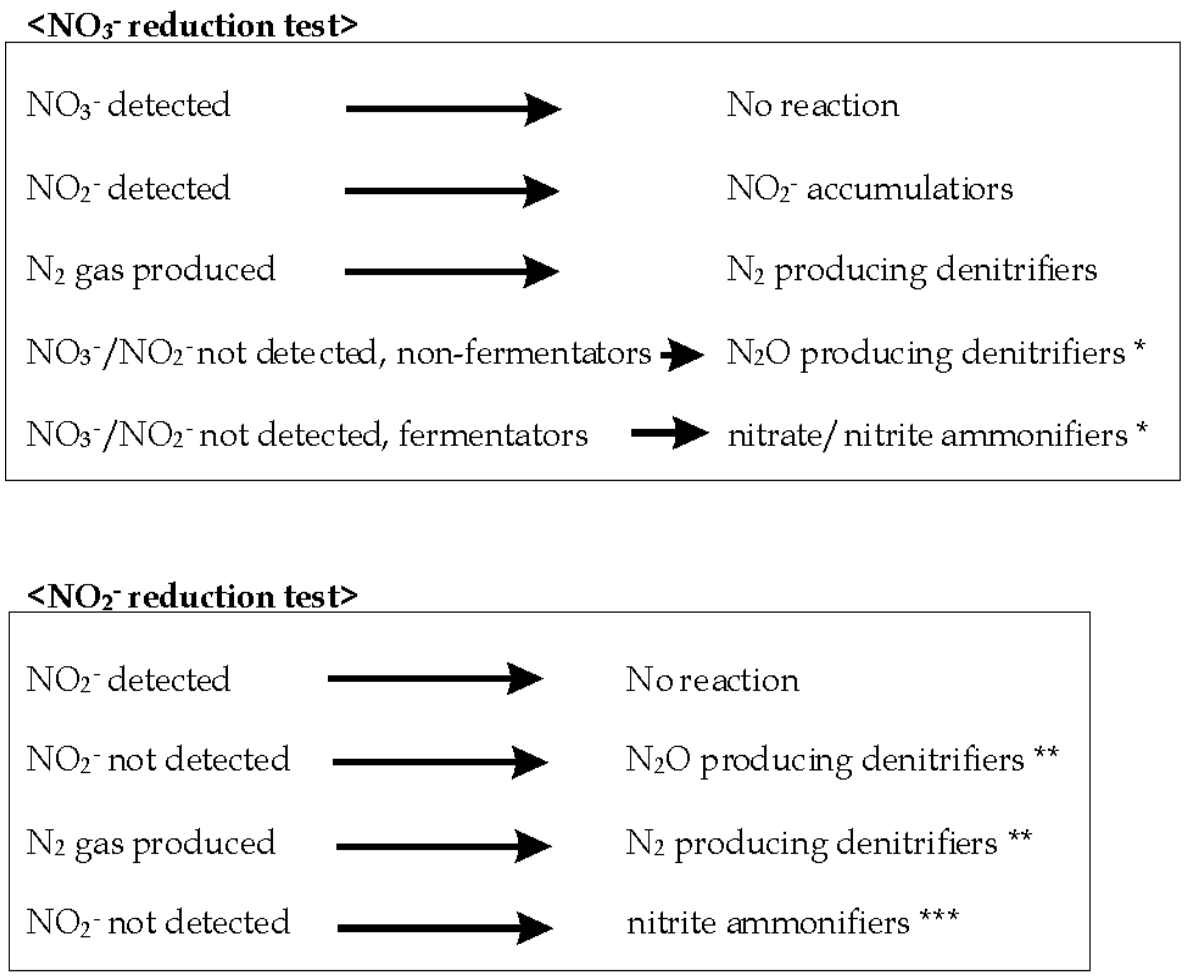


Figure 2-2 A scheme for screening denitrifying and ammonifying bacteria

2.5 Identification of the isolates

The isolates having the ability of reducing nitrate/nitrite were identified by using an ID test (Nissui Pharmacy, Tokyo); the NF-18 kit for non-fermentators and EB-20 kit for fermentators, respectively. Gram stain was done with the Stainer No.1 (Nissui Pharmacy, Tokyo), whilst the oxidase and motility tests were according to the laboratory manual (Tokyo Bureau of Sewerage, 1990).

3. Results and Discussion

3.1 Comparison of the two media

Table 2-2 shows a representative result of the time course of the number of denitrifying bacteria in the activated sludge mixed liquor counted by gas production in two different media. The initial gas production was observed in the lower dilution of the Giltay broth, whilst the gas production was often observed in the higher dilution of the nitrate broth. Even reversed MPN code could be found during the incubation of the nitrate broth. This implied the result of a possible interference between denitrifying and other nitrate-reducing bacteria of competing for the same substrate in a test tube.

However, the final counts obtained by the nitrate broth were higher than those by the Giltay broth. Thus, the nitrate broth seemed to support a wide range of denitrifying bacteria as compared with the nutritionally restricted medium such as Giltay broth (Alexander, 1965). Hence the nitrate broth was employed as a basal medium for further examination.

3.2 Nitrate/nitrite reduction patterns

Table 2-3 summarizes the metabolic patterns of dissimilarity nitrate/nitrite reduction observed in this study. **Table 2-4** shows the frequency of the bacterial isolates that catalyzed the distinct metabolic patterns listed in **Table 2-3**. The percentage was calculated based on the numbers of each metabolic pattern found in the total 50 isolates of each sample.

Regarding the bacterial counts in various water samples, highest numbers of 10^7 CFU/ml were obtained from raw sewage (120 mg/l BOD) and activated sludge, equivalent to the counts from the heavily polluted Sakagawa River.

Table 2-2 Time course in the number of denitrifying bacteria in the MPN tubes

Incubation time (days)	Giltay broth	Nitrate broth
3	3-0-0*	1-5-1*
5	5-2-0	3-5-1
7	5-2-0	4-5-1
10	5-3-0	4-5-1
18	5-3-0	5-5-1
20	5-3-0	5-5-1
Final counts	7.9×10^5	3.5×10^6

** Figures indicate the number of positive tubes of gas production as the MPN code.*

*** Activated sludge mixed liquor sample was used for this preliminary examination.*

Table 2-3 Metabolic patterns of dissimilatory nitrate/nitrite reduction

Type	Description
I	Nitrate reduction, *nitrite accumulating*
II	Nitrate ammonification, *ammonia accumulating*
IIIa	Nitrate denitrification, *nitrous oxide accumulating*
IIIb	Nitrate denitrification, *dinitrogen gas producing*
IV	Nitrite ammonification, *ammonia accumulating*
Va	Nitrite denitrification, *nitrous oxide ammulating*
Vb	Nitrite denitrifying, *denitrogen gas producing*

The bacterial counts of other samples were in the order of 10^5 CFU/ml, except for the count of 10^4 CFU/ml in the sample collected at the Hamura Bridge in the less polluted, upper Tamagawa River; this indicating a close relationship between heterotrophic bacterial counts and the extend of organic pollution.

Table 2-4 shows the result of metabolic patterns of dissimilatory nitrate-nitrite reduction. As for the frequency of the nitrate/nitrite respiring bacteria found in this study, 66% and 34% of the total isolates were able to reduce nitrate and nitrite, respectively. Interestingly, the isolates capable of accumulating nitrite from nitrate tended to accumulating ammonia from nitrite when they were incubated with nitrite instead of nitrate. This suggested that the same strain could show the different metabolic patterns, depending on their conditions of mixed culture and the actual environment.

The nitrite accumulating isolates (Type I) were often found in the samples of sewage and polluted river water, accounting for 50-60% predominance, whilst the percentage of the less polluted site at the Hamura Bridge was low (22%). The ammonia accumulating isolates from nitrate (Type II) were generally scarce, but those from nitrite (Type IV) were around 15-30% of frequency in the sewage-related samples. None of ammonia accumulating bacteria (Types II and IV) was isolated from the polishing creek receiving treated sewage effluent. However, there was no correlation between the isolation frequency of ammonia accumulating bacteria and the degree of water pollution, *i.e.* lower (Type II, 0%; Type IV, 2%) at the down stream and higher (Type II, 6%; Type IV, 10%) at the upper steam of the Tamagawa River.

The nitrous oxide producing isolates were more frequently detected in the

Table 2-4 Metabolic patterns of the isolates utilizing nitrate/nitrite broth

Samples Type	I	II	IIIa	IIIb	IV	Va	Vb
1 Raw sewage	62	4	0	14	26	10	10
2 Settled sewage	50	4	4	24	14	12	26
3 Activated sludge A	38	0	6	16	6	10	12
4 Activated sludge B	50	2	8	14	2	18	12
5 Artificial creek	52	0	2	16	0	6	20
6 Hayase Bridge*	32	12	8	8	20	20	4
7 Tokumaru Bridge*	54	0	8	14	4	16	10
8 Ippei Bridge**	44	0	4	14	4	14	18
9 Chofu Bridge***	44	0	2	12	2	10	12
10 Hamura Bridge***	22	3	0	10	10	0	12

** Shingashi River, ** Sakagawa River, *** Tamagawa River* (Frequency, %)

nitrite broth than the nitrate broth. None of the nitrous oxide producers was isolated from the upper stream of the Tamagawa River.

The frequency of nitrogen gas production was 4-26% of each isolates, being usually abundant in the settled sewage contaminated with activated sludge and the heavily polluted river water from the Sakagawa River.

The overall presence of nitrite-dependent denitrifiers seemed to be up to 2-4%, excepting for the maximum value of 10% of frequency in the polishing creek sample, as shown in **Table 2-5.** Obviously, the nitrate broth supported a larger number of denitrifying bacteria, as compared with the Giltay broth.

Table 2-5 Gas and ammonia production in the Giltay and nitrate/nitrite broth

Samples	Giltay[a]	Nitrate[a]	Nitrite[a]	NH_4^{+}[b]
1 Raw sewage	0	14	0	22
2 Settled sewage	10	24	2	10
3 Activated sludge A	2	16	2	6
4 Activated sludge B	4	14	2	0
5 Artificial creek	0	16	10	0
6 Hayase Bridge*	2	8	0	8
7 Tokumaru Bridge*	4	14	4	4
8 Ippei Bridge**	2	14	4	4
9 Chofu Bridge***	0	12	2	2
10 Hamura Bridge***	6	10	2	0

** Shingashi River, ** Sakagawa River, *** Tamagawa River* (Frequency, %)

[a] Gas production from broth, [b] Ammonia produced from nitrite but not from nitrate broth.

3.3 Generic composition of nitrate-reducers

A total of 184 nitrate/nitrite reducing isolates were identified using the above-mentioned kits, as summarized in **Table 2-6**. A total of 84 isolates were identified as *Pseudomonas* spp., whilst only 16 isolates were identified as *Alcaligenes* spp. The other isolates capable of nitrogen gas production were tentatively identified as the following genera such as *Acinetobacter*, *Moraxella*, *Flavobacterium* and *Alteromonas*. Some species, other than *A. denitrificans*, A. *faecalis*, *P. aeruginosa* and *P. fluorescens*, have not been so far accepted as true

Table 2-6 Generic composition of denitrifying and ammonifying bacteria

Samples	#1	2	3	4	5	6	7	8	9	10
Acinetobacter	1		1				1			
Moraxella	3	1	3	2			1	3	2	
Flavobacterium	4			1	3	1			2	1
Alcaligenes	4	1		4	5	3	1	1	1	1
Pseudomonas	4	5	7	10	6	9	11	14	8	5
Alteromonas						2	2			
Aeromonas	6	13	2	1		8	2	2	1	1
Klebsiella	2									
Escherichia	1									
Vibrio							7			3
Not identified		1		2		2				1
No of isolates	26	23	16	20	14	24	18	19	13	11

denitrifiers. This may be due to the limitation of the kits which were originally designed for a diagnostic purpose of medical materials. In using these kinds of commercial products, the identification codes (*i.e.* biochemical reaction profiles) should be modified in advance, so as to meet the target bacteria isolated from other habitats such as river water and sewage materials.

A total of 10 isolates out of 44 isolates of the fermentative bacteria turned out denitrifiers that produced gas in the nitrate broth, *i.e.* eight strains belonged to *Aeromonas* and two belonged to *Klebsiella*.

Table 2-7 Comparison of generic compositions of denitrifiers isolated using the Giltay and nitrate broth

Species	Giltay broth	Nitrate broth
Acinetobacter spp.	0	1
Moraxella spp.	0	4
Flavobacterium spp.	0	4
Alcaligenes denitrificans	1	3
A. faecalis	1	10
A. putrefaciens	1	2
A. xylosoxidans	0	2
Pseudomonas aeruginosa	3	9
P. fluorescens	2	2
P. acidvorans	1	4
P. alcaligenes	0	3
P. diminuta	0	13
P. paucimobilis	0	3
P. pseudoalcaligenes	1	1
P. vesicularis	2	5
Alteromonas spp.	1	1
Aeromonas spp.	1	2
Klebsiella spp.	1	1
Not identified	0	1
No. of isolates	15	71

Other ammonia accumulating isolates were tentatively identified as the following genera such as *Aeromonas*, *Klebsiella*, *Escherichia* and *Vibrio*. However, the rest six isolates were not clearly identified using the kit, as their codes did not match to those in the existing database.

Table 2-7 shows the comparison of generic composition of denitrifying isolates using the Giltay and nitrate broth. The results suggested that the Giltay broth restricted the growth of a wide range of taxonomic groups of denitrifiers, due to the difference in ingredients of two media, maybe reflecting a possible selection by substrates. **Table 2-8** lists the generic composition of the nitrite dependent denitrifiers and ammonifiers obtained in this study. Most of the nitrite-dependent ammonifiers were grouped as *Aeromonas* spp.

Table 2-8 Generic composition of the isolated nitrite dependent denitrifiers and ammonifiers

Denitrifiers		Ammonifiers	
Moraxella spp.	1	*Aeromonas* spp.	23
Flavobacterium spp.	2	*Escherichia* spp.	1
A. faecalis	3	*Vibrio* spp.	2
A. putrefaciens	1	Others	2
P. alcaligenes	1	No. of isolates	28
P. diminuta	2		
P. vesicularis	4		
No. of isolates	14		

3.4 Ecological implications of competitive nitrate reduction

Nitrate reducers and fermentators are considered to be versatile microorganisms that can grow under anaerobic conditions, respectively utilizing nitrate and sugars as an energy source. The population dynamics of these kinds of bacteria may be influenced by the substrate supplied and the other environmental conditions. In particular, denitrifying and ammonifying bacteria compete with each other for nitrate as their common substrate. Such competition can be observed even in a test tube of the MPN enumeration.

Past trials of determining the metabolic patterns of nitrate reduction have been carried out in marine (Kimata *et al*., 1968) and soil (Gamble *et al*., 1977) environments. In the former investigation, five distinct patterns were decided by observing whether gas was produced or not. They judged that the result of no gas production after nitrate/nitrite consumption was regarded as ammonia production. However, no gas production could also imply the possibility of nitrous oxide production, because of its higher degree of solubility. In this case, no gas might be detected in the Durham tubes during incubation. In this study, putative nitrate ammonifying bacteria were distinguished from the nitrous oxide producers by the OF test, as the fermentative bacteria could be responsible for nitrate ammonification.

The nitrate reducing bacteria, including denitrifying members, were abundant in organically polluted waters (Takeuchi and Hata, 1985). The similar tendency was confirmed in this study, thereby the colonization, higher organic and nitrate contents presumably enabled to accumulate the nitrate reducing and denitrifying bacteria.

Nitrite is an intermediate product, which is unstable and toxic to other microorganisms. The presence of nitrite may induce the enzyme of nitrate reduction based on the present observation. It could also be regarded as a sort of detoxification against nitrite.

None of nitrate ammonifiers were detected from the highly matured effluent of sewage origin. This result suggested that the balance of organic matter and nitrate seems to determine whether denitrifying or ammonifying bacteria tended to predominate. It would be reasonable that fermentative bacteria such as enterobacteriaceae demand higher concentrations of organic matter for their growth. Actually, nitrate ammonification surpass denitrification in the sediments of a larger amount of organic matter (Koike and Hattori, 1978).

Some strains may lack the enzyme that catalyzes nitrous oxide reduction; this property could be genetically stable or influenced by the environmental conditions. For example, 77% of the denitrifying isolates were able to produce nitrogen gas from nitrate, whilst only 54% of those showed gas production. Nitrite, and other toxic substances such as cyanide, azide and sulphide, seems to give stress to some denitrifying bacteria (Brown, 1988).

The presence of nitrite dependent denitrifiers was confirmed in this study, but the frequency of isolation was negligible (2-4%). In addition, being different from a pure culture grown in a test tube, naturally occurring bacteria are basically mixed culture. Thus, even if the nitrite dependent denitrifiers exist in nature, they may be supplied with nitrite from the other co-existing nitrate reducing bacteria, as a result of commensalisms.

In order to meet an appropriate sewage treatment process to the receiving

environment, evaluation of possible nitrate reduction to ammonia should be considered in advance, so as not to waste energy to facilitate nitrification within the process. An examination on the nitrate reducing bacterial flora responsible for denitrification and ammonification may be useful to predict the fate of nitrate removed to the air or remained in the water in the receiving environment after the effluent is discharged from the nitrified aeration basins.

References

Alexander M (1965) Denitrifying bacteia, *Methods of Soil Analysis,* Part 2, ed Black CA *et al*., 1484-1486, American Soc Agronomy, Madison.

Brown CM (1988) Nitrate metabolism by aquatic bacteria, *Methods in Aquatic Bacteriology*, ed Austin B, 367-388, John Wiley & Sons, Ltd, New York.

Cole JA, Brown CM (1980) Nitrite reduction to ammonia by fermentative bacteria: a short circuit in the biological nitrogen cycle. *FEMS Microbiol Lett*, **7**: 65-72.

Gamble TN, Betlach MR, Tiedje JM (1977) Numerically dominant denitrifying bacteria from world soils. *Appl Environ Microbiol*, **33**: 926-939.

Jeter RM, Ingraham JL (1981) The denitrifying prokaryotes, *The Prokaryotes – A Handbook on Habitats, Isolation, and Identification of Bacteria*, ed Starr MP *et al*., 913-925, Springer-Verlag, Berlin.

Kimata M, Yoshida Y, Taniguchi M (1968) Studies on microorganisms utilizing inorganic nitrogen compounds in seawater- I Denitrifying bacteria. *Bull Jpn Soc Sci Fish*, **34**: 1114-1117.

Knowles R (1982) Denitrification. *Microbiol Review*, **46**: 43-70.

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

Nakajima T (1982) Distribution of denitrifying bacteria and its controlling factors in freshwater environments. *Jpn J Limnol*, **43**: 17-26.

Partiquin DG, Knowles R (1974) Denitrifying bacteria in some shallow-water sediments: enumeration and gas production. *Can J Microbiol*, **20**: 1037-1041.

Stanier RY, Ingraham JL, Wheelis ML, Painter PR (1986) *The Microbial World*, 5th ed, Pretice-Hall, New Jersey.

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

Sugahara I, Hayashi K, Kimura T (1986) Distribution and generic composition of denitrifying bacteria in coastal and oceanic waters. *Bull Jpn Soc Sci Fish*, **52**: 497-503.

Takeuchi J and Hata Y (1985) Distribution of bacteria associated with various sizes of particulate matter in Harima-nada and Hiuchi-nada areas, Seto Inland Sea. *Jpn J Ecol*, **35**: 49-56.

Terai H (1979) Taxonomic study and distribution of denitrifying bacteria in Lake Kizaki. *Jpn J Limnol*, **40**: 81-92.

Tokyo Bureau of Sewerage (1990) *Microbes in Aeration Tanks-A Guide for Microscopy and Cultivation*, JSWA, Tokyo, 324pp.

Vangnai S, Klein DA (1974) A study of nitrite-dependent dissimilatory micro-organisms isolated from Oregon soils. *Soil Biol Biochem*, **6**: 335-339.

Chapter 3

Bacterial community dynamics in nitrate-fed activated sludge

Abstract

A two-year survey of bacterial populations in activated sludge was carried out in a municipal sewage treatment plant receiving industrial wastewater having a high nitrate content. Phosphorus removal was not improved with a modified operation with a dissolved oxygen (DO)-deficient selector. In these circumstances, the anoxic selector in the aeration tanks acted as a site for denitrification not as a site for phosphorus incorporation. The ecological interactions between the different bacterial species are thought to occur in the anoxic zone. Possible competition between *Psuedomonas/Alcaligenes* group and the *Acinetobacter/Moraxella* group was suggested under laboratory conditions. Commensalism between the *Enterobacter/Aeromonas* group and the *Acinetobacter/Moraxella* group was observed by using a Nuclepore membrane-dialysis culture technique.

1. Introduction

It is well known that various bacteria play important roles in the transformation of organic and inorganic matter in activated sludge as well as in the natural ecosystem. The purification ability of the activated sludge process is usually evaluated by the change of water quality between inflow and outflow, i.e., the nutrient removal ratio. Thus, ecological studies on the bacterial populations in activated sludge are insufficient.

With a minor modification of setting an anaerobic zone in the aeration tanks, biological nitrogen and phosphorus removal is one of the most attractive activated-sludge processes. It is expected to reduce the discharge of nutrients and prevent eutrophication of natural waters. To establish operational conditions to achieve a more stable water quality, the ecological and physiological significance of such a DO-deficient zone should be studied.

Since the discovery of an obligate aerobe, *Acinetobacter* as a phosphorus-accumulating bacterium by Fuhs and Chen (1975), the mechanism of predominance of the specific bacterial group in activated sludge with an anaerobic selector has been noted. In this study, attention was focused on the fluctuation of the bacterial numbers and flora and the selective effect of the DO-deficient zone on the bacterial populations. Improvement of phosphorus removal was not accomplished as expected, however. This paper describes the dynamics of bacterial populations and the influence of nitrate on the selection of bacterial flora by additional laboratory experiments. The possibility of interaction between each bacterial group for their growth and survival in the DO-deficient environment was suggested.

2. Materials and Methods

2.1 Description of full-scale plant studied

Shingashi Treatment Plant, having a treatment capacity of 70,5000 m3/day, is characterized by the industrial effluents containing nitrate. The activated sludge process consists of eighteen 8,428 m^3 (140 x 8.6 x 7.0 m deep) aeration tanks. During the experiment of setting an anoxic selector on the sewage

inflow side, the air diffusers were reduced to minimize circulation of the mixed liquor and prevent sedimentation of the activated sludge. The retention time of the anoxic zone was about 3 hours, half of the total aeration time.

2.3 Enumeration of bacterial populations

Mixed liquor samples were collected from the final end of the aeration tank, and dispersed by blending treatment (16,000 rpm, 5 min). The dispersed samples were subsequently diluted with sterile tap water containing 1 ppm Tween 80 (Jones and Jannasch, 1959). Viable counts of aerobic heterotrophic bacteria were obtained by the spread plate technique using Sakurai's agar (Takii and Konda, 1981). Nitrifying bacteria, denitrifying bacteria, and sulphate-reducing bacteria were enumerated by the most probable number method using Kawai's ammonium or nitrite broth with quartz sand (Kawai, 1969), Nakajima's nitrate broth with Durham tubes (Nakajima, 1979), and a modified ISA semi-liquid medium (Mara and Williams, 1970), respectively.

2.4 Identification of aerobic heterotrophic bacteria

To clarify the flora profile, 50 colonies were taken randomly form each of the agar plates, and purified by streak plating. Then, the isolates of bacteria were roughly grouped according to the scheme of Cowan and Steel (Cowan, 1974). The bacteriological examinations for identification were as follows: Gram reactions, morphology, pigmentation, oxidative and fermentative test (glucose), oxidase test, motility, oxygen requirement, endospore formation, and hydrolyses of casein and starch.

2.5 Laboratory experiment using batch culture

The effects of nitrate and acetate on the changes of bacterial flora were

examined. The activated sludge samples were anaerobically incubated in 200 ml of glass bottles in which nitrate (10 mM $NaNO_3$) or acetate (20 mM CH_3COONa) was added. After 7 days incubation, an aliquot of the sample was spread on Sakurai's agar plates. As previously described, 50 colonies were isolated from each of the agar plates, then identified using the same scheme. Their denitrifying activities were also detected using the previously mentioned nitrate broth.

2.6 Laboratory experiment using dialysis culture

To simulate the interactions between the bacterial groups in an anoxic environment, the following bacterial strains isolated from the *in situ* activated sludge were use: *Pseudomonas* sp. (a glucose oxidative, nitrogen gas producing from nitrate broth, motile short rod), *Acinetobacter calcoaceticus* (an oxidase negative, non-motile cocci rod), and *Aeromonas hydrophila* (a glucose fermentative, gas producing from glucose, motile cocci rod). The above identification were made using Nissui's rapid identification kits (Nussui Pharmacetical Co., Ltd., Japan), NF-18 for non-fermentators and EB-20 for fermentatiors, respectively.

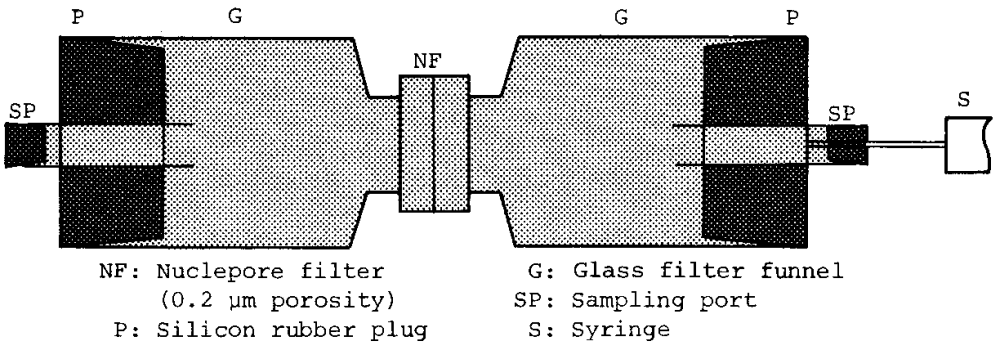


Figure 3-1 A Nuclepore membrane-dialysis culture chamber

Figure 3-1 shows the apparatus for dialysis culture. A Nuclepore filter is said to be the most suitable membrane for dialysis culture because of the rapid permeation of dissolved matter and the scarce attachment of microorganisms (Watanabe, 1987).

3. Results

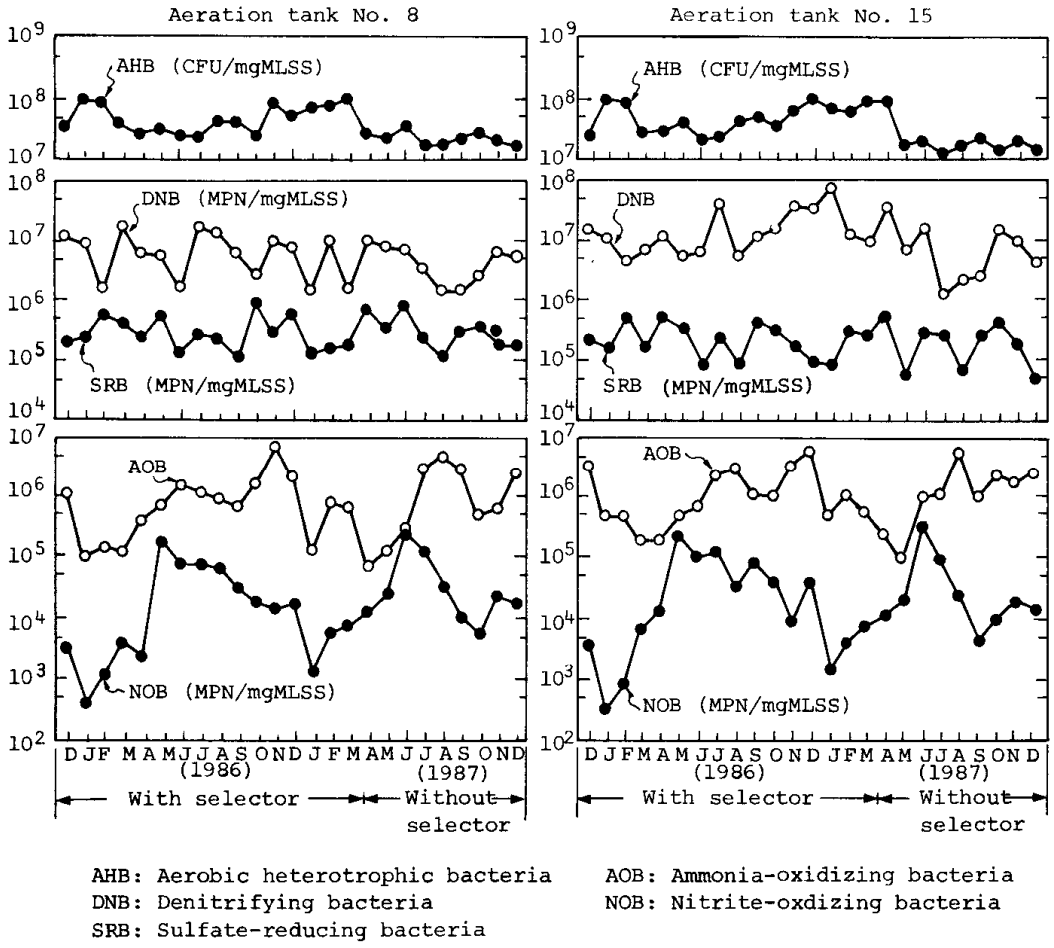
3.1 Fluctuation of bacterial populations

Figure 3-2 shows the seasonal changes in the number of five different bacterial populations in the mixed liquor of activated sludge. There was a tendency for higher numbers of aerobic heterotrophic bacteria in the high water temperature period than in the low water temperature period. In addition to the seasonal changes, the population size of the bacteria was lower under the modified operation with the anoxic selector than under the conventional operation.

There was no difference in the numbers of other bacteria with or without the selector. The population sizes of denitrifying and sulphate-reducing bacteria were respectively 10^6-10^7 MPN/ml and 10^5 MPN/ml. Nitrifying bacteria showed marked seasonal changes, probably depending on the water temperature (12-28^{o}C). As a result, the DO-deficient condition had no effect on the bacterial population changes except for total aerobic heterotrophic counts.

Figure 3-3 shows the changes in the generic compositions of aerobic heterotrophic bacteria in the activated sludge during the operation with and without the selector. Generally speaking, the *Pseudomonas/Alcaligenes* group tended to predominate. Up to 40% of the isolates belonged to the

Acinetobacter/Moraxella group with the selector, however.



MLSS, Suspended solids in the mixed liquor of activated sludge

Figure 3-2 Fluctuation of bacterial populations in activated sludge

3.2 Water quality and removal ratio

Figure 3-4 shows the concentrations of total phosphorus in the filtrate of the mixed liquor at the initial and final sites of aeration tanks. The differences in the concentrations indicate the characteristics of phosphorus release, and the low level of the concentration at the final site indicates a high phosphorus removal ratio. After changing from the operation with the anoxic selector to

the conventional operation, no remarkable release of phosphorus was observed. It is thought that the characteristic activated sludge, having the capability to release phosphorus, was developed during the operation with the DO-deficient selector.

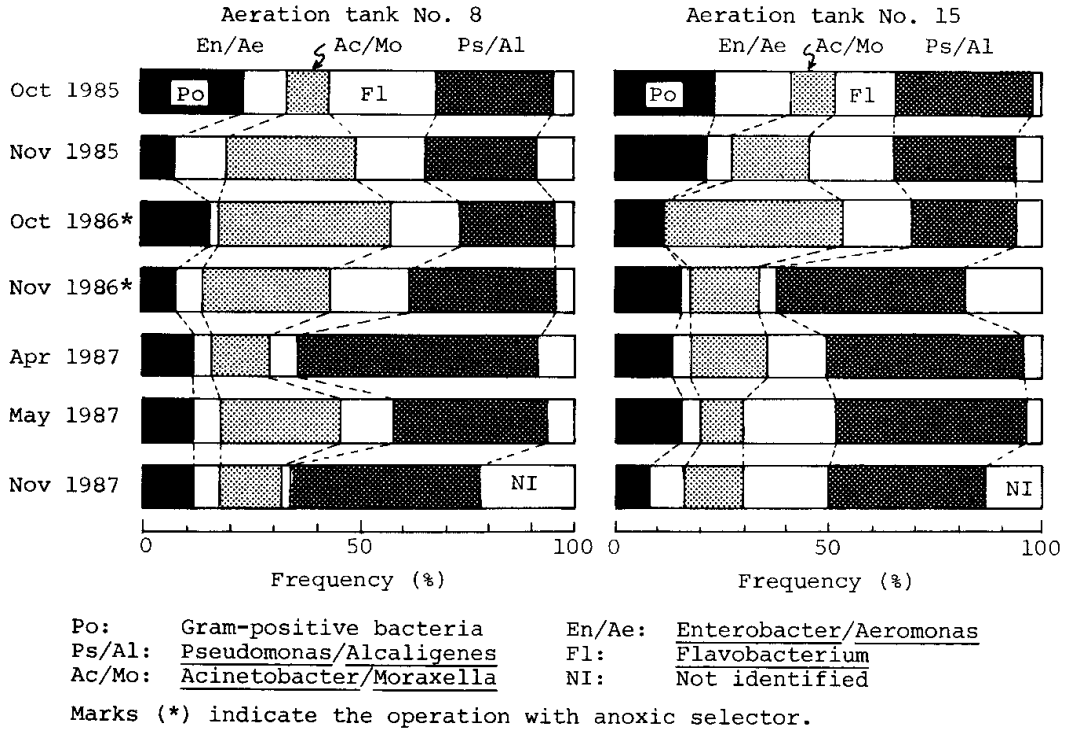


Figure 3-3 Changes in generic composition of heterotrophic bacteria

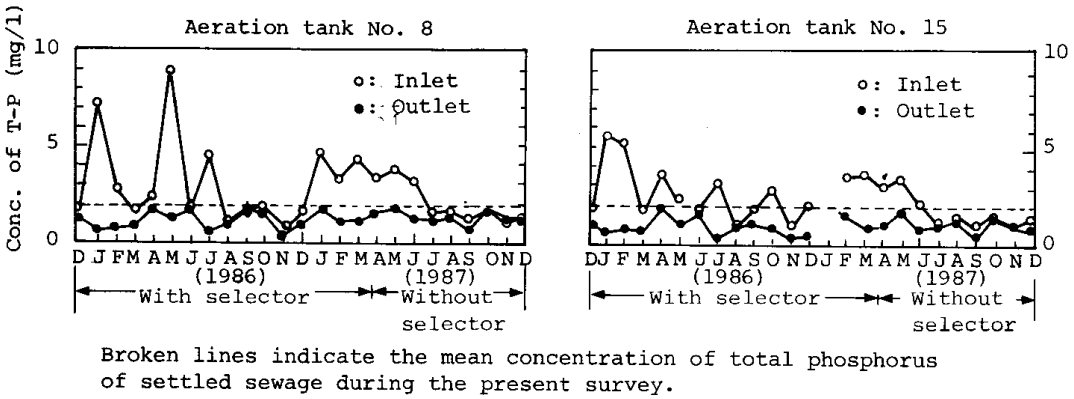


Figure 3-4 Changes in total phosphorus content in the filtrate

TABLE 1 Operational Condition and Resultant Removal Ratio

Operation		Without selector	With Selector	Without Selector
Period		Oct 1984 to Nov 1985	Dec 1985 to Mar 1987	Apr 1987 to Mar 1988
MLSS (mg/l)		1,592 (1,821 - 1,276)	1,929 (2,174 - 1,603)	1,465 (1,773 - 1,200)
MLDO (mg/l)	Head	1.0 (1.7 - 0.7)	0.5 (0.8 - 0.2)	0.8 (1.1 - 0.5)
	End	2.7 (4.4 - 1.2)	3.2 (6.0 - 1.6)	1.4 (3.7 - 0.8)
Inlet, BOD		108 (193 - 62)	113 (180 - 60)	92 (130 - 72)
Outlet, BOD		9 (18 - 5)	12 (24 - 6)	10 (19 - 2)
Removal (%)		91.1 (95.6 - 87.0)	88.4 (96.7 - 82.0)	82.1 (97.2 - 80.2)
Inlet, T-N		39.6 (48.6 - 31.2)	40.8 (59.2 - 23.8)	35.4 (47.3 - 24.5)
Outlet, T-N		28.1 (29.9 - 23.9)	22.7 (34.1 - 12.4)	23.8 (32.0 - 16.3)
Removal (%)		28.6 (40.6 - 18.6)	44.4 (57.9 - 29.2)	32.0 (54.5 - 17.5)
Inlet, T-P		2.8 (3.2 - 2.1)	3.4 (5.4 - 1.8)	2.9 (4.3 - 1.5)
Outlet, T-P		1.3 (1.9 - 0.8)	1.4 (2.4 - 0.7)	1.5 (2.4 - 0.5)
Removal (%)		53.2 (72.4 - 33.3)	56.4 (72.2 - 45.2)	48.9 (81.6 - 35.7)

Filtrate filtered with a No. 5C filter paper was examined.
The figures indicate the mean value (max. - min.).
T-N: Total nitrogen T-P: Total phosphorus

Table 3-1 Operational conditions and resultant removal ratios

Table 3-1 outlines the operational conditions and resulting removal ratio of nutrients. The data show a tendency for the nitrogen and phosphorus removal ratio to be more or less improved by setting up the anoxic selector. Phosphorus removal was not remarkably improved, as expected, however.

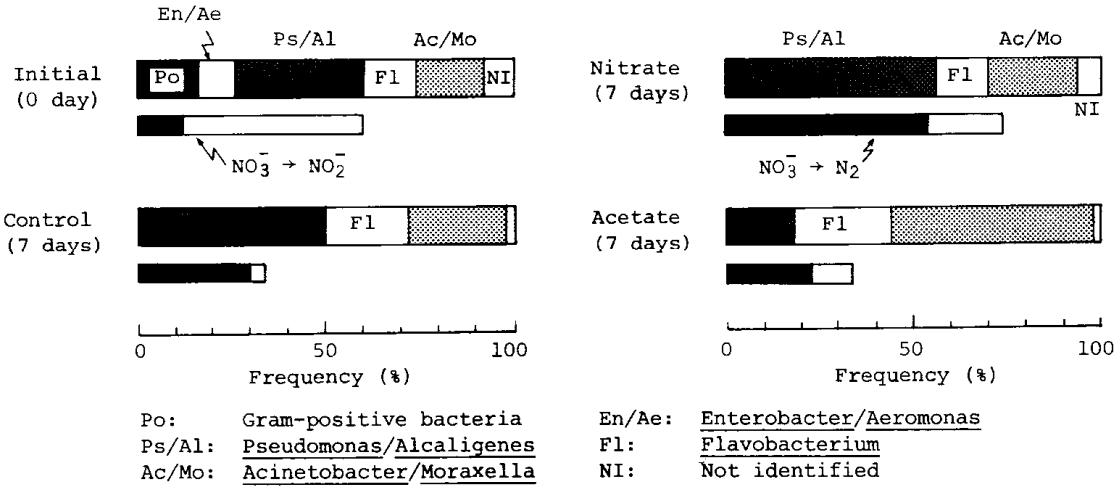


Figure 3-5 Effects of nitrate and acetate on succession of heterotrophic bacterial flora

3.3 Succession of bacterial flora with acetate and nitrate

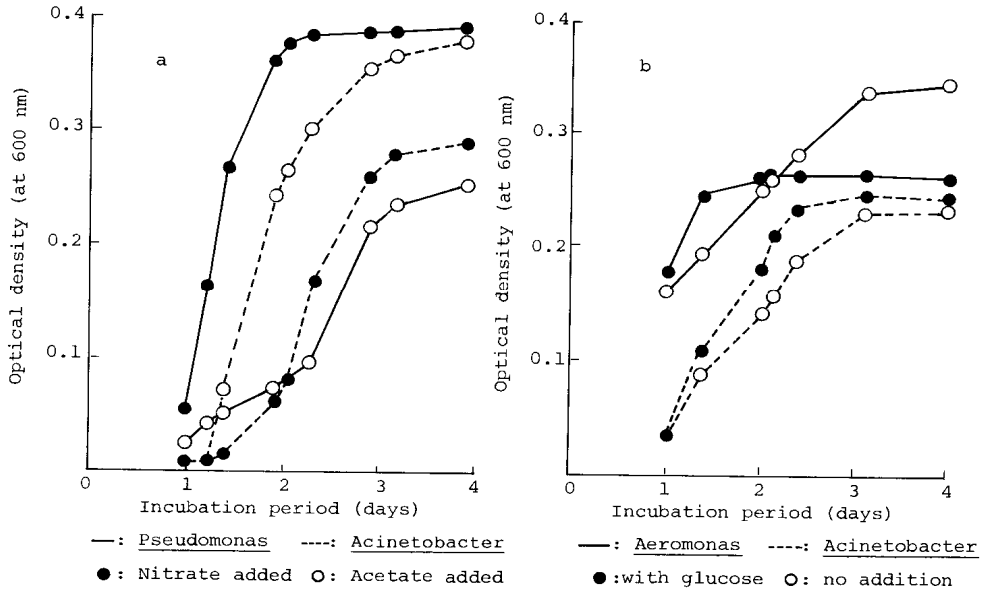
Figure 3-5 shows the results of bacterial succession during the batch culture experiment for clarifying the effects of acetate and nitrate on the bacterial flora in an anaerobic environment. The initial and final floras of aerobic heterotrophic bacteria had diverse generic compositions. The *Enterobacter/Aeromonas* and the Gram positive groups disappeared after the enrichment culture. The *Acinetobacter/Moraxella* group predominated in the mixed culture fluid with acetate, and the *Pseudomonas/Alcaligenes* group predominated in that with nitrate. Moreover, 54% of the isolates from the culture fluid with nitrate had denitrifying activity. The glucose fermentative *Enterobacter/Aeromonas* group was present in only the initial environment.

3.4 Simulated experiments on competition and commensalism

Figure 3-6 shows the representative interactions between the bacterial species. **Figure 3-6a** shows the growth of *Pseudomonas* was rapid, whereas the initial growth of *Acinetobacter* was delayed for a day in an anaerobic environment with nitrate added, which was more favourable for *Pseudomonas*. Conversely, the growth of *Acinetobacter* surpassed that of *Pseudomonas* when acetate was added. *Pseudomonas* and *Acinetobacter* showed a competitive relationship with each other. Whether *Pseudomonas* or *Acinetobacter* predominated in the monoxenic cultures depended on whether nitrate or acetate was added.

As **Figure 3-6b** shows, the relationship between *Aeromonas* and *Acinetobacter* was somewhat of a commensalism. The initial rapid growth of *Aeromonas* and the subsequent inhibitory effect by the metabolites were observed under

the condition cultured with glucose as a fermentation substrate. As compared with the control without glucose, the growth of *Acinetobacter* was stimulated by the coexistence of *Aeromonas* and the addition of glucose; that is, by the organic acids provided by *Aeromonas*.



(a. competition, b. commensalisms)

Figure 3-6 Interactions of bacterial species simulated using a dialysis culture

4. Discussion

According to this study, phosphorus removal is not improved by using a modified operation with a DO-deficient zone. The reason is considered that the anoxic environment was created by simply reducing the aeration strength. Furthermore, the receiving of industrial wastewater having high nitrate content should be considered. Hashimoto and Furukawa (1984) reported that biological phosphorus release is inhibited until nitrate is biologically removed under laboratory conditions. This means that the anoxic selector with nitrate

may act primarily as the site for denitrification rather than for phosphorus incorporation. Actually, the mean concentration of nitrate (NO_3-N) at the end of the anoxic zone was 0.3 mg/l (max. 8.0 mg/l; min. 0.0 mg/l) under the operation with the selector, whereas that at the same site was 4.1 mg/l (max. 12.0 mg/l; min. 0.6 mg/l) under the conventional operation. This difference is thought to be due to bacterial denitrification.

As previously described, the anoxic selector played a more important role in nitrogen removal than in phosphorus removal. Although the removal ratios were not revealed, there was evidence of specific activated sludge being formed, showing the property of phosphorus release and biological nutrient removal from the viewpoint of microbial ecology.

A design manual on biological phosphorus removal published by U.S. EPA (Bowker and Stensel, 1987), points out that the essence of the modified activated sludge process is that selection of specific microorganisms in the mixed culture. In other words, the process consists of the competition of microorganisms and the resulting predominance of phosphorous-accumulating microorganisms. Inamori *et al.* (1986) found that there is no difference in the protozoan fauna by repeating the anaerobic and oxic conditions. This study indicated that there was little difference in the population sizes of obligate aerobes, facultative anaerobes, and obligate anaerobes by setting up the anoxic selector. Only a difference in the number of total aerobic heterotrophic bacteria was observed. This means that the difference may be due to their generic compositions.

Acinetobacter has been accepted as a major agent in the process of biological phosphorous removal since the report by Fuhs and Chen (1975). A

non-motile, short, almost spherical rod, *Acinetobacter*, in general, is characterized by slow multiplication and weak decomposing activity of organic matter in natural habitats, as compared with other saprophytic bacteria such as *Pseudomonas*. *Acinetobacter* is an obligate aerobe; however, it has a useful capability for surviving in an anaerobic environment. *Acinetobacter* is at a disadvantage in an anaerobic environment; however, it can grow by synthesizing poly-β-hydroxybutyrate (PHB) from acetate while hydrolyzing the reserved polyphosphate. Therefore, there is no possibility of bacteria other than *Acinetobacter* predominating in an anaerobic environment. In this respect, the anaerobic zone acts as a selector for the enrichment of the specific bacterial group having the same survival strategy as *Acinetobacter*.

What can have happened microbiologically, where there is an anaerobic zone receiving an inflow having high nitrate content? Denitrifying bacteria usually grow by ordinary respiration in an aerobic environment, whereas they can grow anaerobically in the presence of nitrate as their electron acceptor. **Figure 3-6** shows there is a competitive relationship between *Pseudomonas* and *Acinetobacter*. Nitrate stimulates the growth of *Pseudomonas*, whereas acetate supports the growth and survival of *Acinetobacter*. The enrichment of *Acinetobacter* in an anaerobic environment with acetate added was reported by Dunn *et al.* (1980) in incubating estuarine sediment slurry. The influence of nitrate on bacterial competition is thought to account for the failure of *in situ* predominance of *the Acinetobacter/Moraxella* group in the modified activated sludge process with the DO-deficient selector.

Brodisch (1985) stated that *Aeromonas* enhanced the phosphorus uptake of

Acinetobacter; he thought of the anaerobic zone as a fermentative reactor. Figure 3-6 shows there seems to be a commensal relationship between *Aeromonas* and *Acinetobacter*. The former produces a fermentative product like acetate, whereas the latter presumably consumes it for growth and survival in an anaerobic environment. The percentage of the fermentative *Enterobacter/Aeromonas* group was relatively low in a real plant where acetate might to be derived directly from inflows.

There is another opinion that a wide variety of bacterial genera other than *Acinetobacter* can accumulate polyphosphate; for example, *Enterobacter*, *Rhodobacter*, *Mycobacterium*, *Corynebacterium*, etc. In addition, the cells of *Acinetobacter* hardly flocculated and settled downward in pure culture experiments conducted by Mino *et al.* (1986). An increase of bacterial populations suitable for the treatment is obtained by recovering the floc of activated sludge at the secondary clarifiers and recycling it to the head of aeration tanks.

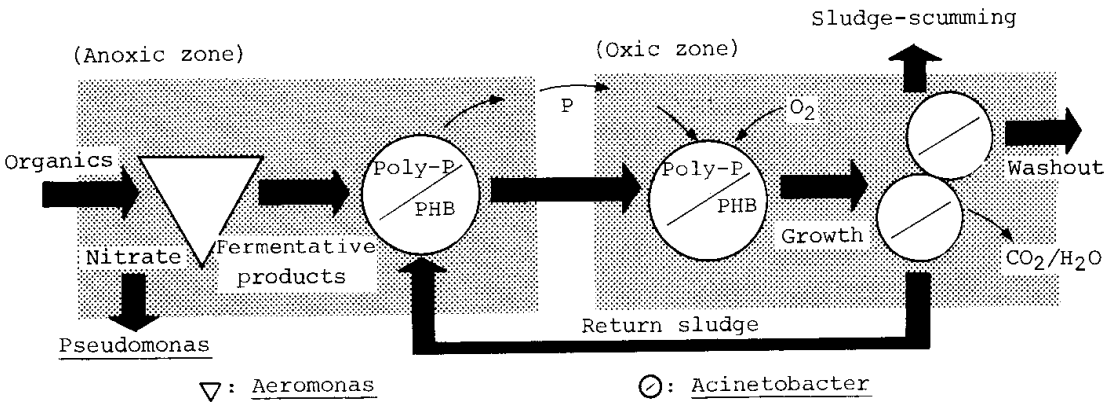


Figure 3-7 Possible mechanism of predominance of *Acinetobacter* or *Pseudomonas* in the anoxic selector

Acinetobacter is known as representative free-living bacteria suspended in the over-lying waters of coastal regions (Takeuchi, 1983) and closed bays (Simidu *et al*., 1977). Thus, *Acinetobacter* would not predominate in the plug flow activated sludge process unless their cells are associated with the sludge particles in any ways. Fuhs and Chen (1975) observed that *Acinetobacter*-like cells were embedded in the sludge after repeating the anaerobic and oxic states under laboratory conditions. The attachment mechanism of *Acinetobacter* onto the floc remains unclear.

In addition, the ecological role of fermentative bacteria such as the *Enterobacter/Aeromonas* group should be investigated as the supplier of acetate and consumer of nitrate that could compete for denitrification.

Figure 3-7 shows the mechanism resulting from this study on the predominance of *Acinetobacter* or *Psudomonas* in the anoxic zone. It is important to keep a large biomass of phosphorus-accumulating bacteria for the improvement of phosphorus removal. There remains the possible competition between *Acinetobacter* and nocardioform bacteria (actinomycetes), *i.e*., *Nocardia* and *Rhodococcus*, which can reserve both polyphosphate and poly-β-hydroxybutyrate and cause a severe sludge scumming problem (Lemmer, 1986). In the Shingashi Treatment Plant, it is clear that nocardioform bacteia are plentiful in the mixed liquor and accumulate at the dead space of the activated sludge process. Further studies are needed to clarify the ecological properties of phosphorus-accumulating bacteria in mixed cultures such as activated sludge.

References

Bowker RPG and Stensel HD (1987) Biological phosphorus removal mechanism, *Design Manual: Phosphorus Removal*, U.S. EPA, Cincinnati, pp15-19.

Brodisch KEU (1985) Interaction of different groups of micro-organisms in biological phosphate removal. *Water Sci Tech* **17** (11/12): 89-97.

Cowan ST (1974) *Manual for the Identification of Medical Bacteria* 2nd ed., Kindai Shuppan, Tokyo, pp62-164.

Dunn GM, Wardell JN, Herbert RA, Brown CM (1980) Enrichment, enumeration and characterization of nitrate-reducing bacteria present in sediments of the River Tay Estuary. *Pro Roy Soc Edinb* **78B**: s47-s56.

Fuhs GW, Chen M (1975) Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. *Microb Ecol* **2**: 119-138.

Hashimoto S, Furukawa K (1984) Biological phosphorus release from activated sludge of sludge recycling nitrification-denitrification process. *J Ferment Technol*, **62**: 437-444.

Inamori Y, Takahashi T, Sudo R (1986) The effect of anaerobic conditions on activated sludge process. *J Jpn Sewage Works Assoc*, **23** (5): 61-69.

Jones GE, Jannasch HW (1959) Aggregates of bacteria in seawater as determined by treatment with surface active agents. *Limnol Oceanogr*, **4**: 269-276.

Kawai A (1969) Enumeration of bacterial populations which metabolize nitrogen compound. *A Manual on Methods for Biological Productivity of Fresh Waters*, JIBP-PF Committee (eds), Kodansha, Tokyo, pp 291-295.

Lemmer H (1986) The ecology of scum causing actinomycetes in sewage treatment plants. *Wat Res*, **20**: 531-535.

Mara DD, Williams DJA (1970) The evaluation of media used to enumerate sulphate reducing bacteria. *J Appl Bacteriol*, **33**: 543-552.

Mino T, Tsuzuki Y, Matsuo T (1986) Pure culture operation of anaerobic aerobic process for biological phosphorus removal. *The 3rd German-Japanese Workshop on Wastewater and Sludge Treatment*, 34pp.

Nakajima T (1979) Denitrificatin by the sessile microbial community of a polluted river. *Hydrobiologia*, **66**: 57-64.

Simidu U, Kaneko E, Taga N (1977) Microbiological studies of Tokyo Bay. *Microb Ecol*, **3**: 173-191.

Takeuchi J (1983) *Studies on bacteria associated with suspended matter in coastal seawater*. MSc thesis, Kochi Univ, 115pp.

Takii S, Konda T (1981) Influence of dilution rate on the bacterial flora of activated sludge origin in continuous mixed culture systems. *Jpn J Ecol*, **31**: 13-22.

Watanabe Y (1987) The use of dialysis culture chamber to measure N/C and P/C ratios of individual phytoplankton species. *Jpn J Limnol*, **48**: 137-140.

Chapter 4

Nitrate ammnifying bacteria in hypernutrified estuarine sediment

Abstract

Distribution and diversity of *nrfA* gene encoding dissimilatory nitrite reduction to ammonium (DNRA) in the sediments of the Colne River, North Essex, UK, were investigated. Sequencing cloned *nrfA* fragments amplified from environmental DNA enabled structure analysis of the bacterial community responsible for this pathway. The DNA was extracted from the sediment samples at different depths from the estuary ranging from freshwater to seawater regions, and amplified using specific PCR primer pairs targeting for the *nrfA* gene. Analysis of the *nrfA* clones showed two distinct clusters corresponding to their origins, namely, divided into the stable sites (marine and freshwater regions) and the unstable sites (brackish water region), where the tidal rise and fall constantly disturbs the environmental conditions. In addition, the *nrfA* clones from the deeper layer of the sediment formed a more homogenous community than those from the surface layer of the sediment. This may be due to more isolated and anaerobic conditions kept in the deeper sediment less influenced by the overlying water and other environmental factors. Most of the *nrfA* clones from the Colne estuarine sediments formed several distinct clusters including known nitrate ammonifiers such as *Aeromonas*, *Shewanella, Desulfovibrio* and *Sulfurospillum*. One of which was, however, related to *Bacteroides* but still quite divergent (~70% identity) and the rest forming unknown clusters of supposedly uncultured members of bacteria.

This is the first trial to describe the *nrfA* partial sequences derived from a natural environment, with reference to their habitat-specific community structure.

1. Introduction

Estuaries are recognised to be a filter (Malone, 1984), receiving a continuous nitrate (NO_3^-) loading of anthropogenic origins such as leaching fertilisers from farm land and discharging effluent from sewage works. The nitrate which penetrates into the bottom sediment is rapidly consumed as an electron acceptor by benthic bacteria under anoxic condition, and the sediment acts as a sink for excess nitrate as one of causative solutes for severe eutrophication (Nedwell *et al.*, 1999). Nitrate discharged into an estuary is supposed to be eventually removed from the water to the atmosphere via denitrification. In this sense, estuaries have been regarded as naturally occurring bioreactors in the environment (Nedwell, 1975; Owens, 1986).

However, epoch-making discoveries were independently but at the same time published by marine geochemists (Koike and Hattori, 1978; Sørensen, 1978), pointing out that nitrate was not always reduced to gaseous nitrogen such as dinitrogen (N_2) and nitrous oxide (N_2O) but returned to ammonium (NH_4^+) remaining soluble in the water. Their findings also suggested that dissimilatory nitrate reduction to ammonium (DNRA), or nitrate ammonification, was equally as important as denitrification, both potentially competing with each other for nitrite (NO_2^-). Therefore, the ecological effect of nitrate ammonification on the environment should not be underestimated

and be recognised as a hidden negative element in evaluating self-purification capacity.

In fact, excretion of ammonium coupled with nitrate respiration in a test tube of *E. coli* culture was noticed by microbiologists in the mid-1950s (Taniguchi *et al.*, 1956). However, its ecological meaning of the rapid accumulation of ammonium *in vitro* had been ignored for a long period by microbiologists themselves (Cole, 1978), which had ended in failure to be deduced from a test tube towards the outer environment. Based on physiological and enzymatic studies, Cole and Brown (1980) gave this missing link a new concept of "a short circuit" in the biological nitrogen cycle.

Since then, a series of ecological studies on nitrate ammonification had been carried out using enrichment cultures of sediment slurry (Dunn *et al.*, 1980) under different conditions of the carbon source, nitrate content and salinity. King and Nedwell (1985) demonstrated using sediment slurry that nitrate ammonification could surpass denitrification in low concentrations of nitrate. Higher temperatures were likely to determine the predominance of nitrate ammonifiers and their end products, which were suggested by a chemostat culture (Ogilvie *et al.*, 1997a) and an autoecological experiment using environmental isolates (DL Llyod, PhD thesis, Essex, 1999). Tiedje *et al.* (1982) pointed out that organic carbon could also act as a selector for the nitrate ammonifiers, drawing an overall perspective of partitioning nitrite into the ammonification and denitrification routes in various habitats.

However, these past studies had been restricted by culture-depending methodology or the relevant activity measurements without information on the

corresponding taxa. Although candidates capable of conducting nitrate ammonification, e.g., fermentative bacteria and other anaerobic bacteria such as *Clostridium* and *Desulfovibrio*, were listed (Tiedje, 1988), it has been still uncertain which types of bacteria exist and actually occupy their ecological niches in the outer environment.

A metagenomic approach, consisting of DNA extraction from the environment, PCR amplification of a specific functional gene and reconstruction of the relevant clone library, enables us to search the gene sequence resources for identifying and analysing the target functional community *in situ*. These approaches have been applied to denitrification-related genes, *nirK/nirS,* coding for nitrite reductases (Braker *et al.*, 1998), *nosZ* for nitrous oxide reductase, (Scala and Kerkhof, 1998), and later *norB,* for nitric oxide reductase, (Braker and Tiedje, 2003) in the bulk DNA isolated from various aquatic sediments. Recently, nitrate ammonification-related gene, *nrfA* (named after Nitrite Reduction by Formate, coding for another dissimilatory nitrite reductase containing cytochrome c552 [EC 1.7.2.2]) has been reported from anaerobic wastewater treatment reactors (Mohan *et al.*, 2004) for the first time, but no information is so far available from any other natural habitat.

The aim of this study was to develop and optimise the *nrfA* specific primer pairs to describe the distribution and phylogenetic status of the nitrate ammonifiers inhabited the sediments of the tidal Colne, a representative model of the hypernutrified estuary, forming a complex of nitrate and salinity gradients. The present results demonstrated clear differences in the habitat-specific community structure that may be reflected by the

environmental constraints. Particularly, the community structure embedded in the deeper sediment was differentiated from that of the surface sediment. Most of the *nrfA* partial sequences from the sediments were most closely related to those of known members of the nitrate ammonifiers such as *Aeromonas*, *Shewanella, Deslufovibrio, Sulfurospirillum* mixed with the anaerobic bioreactor-derived clones, whilst some formed unique clusters grouped with *Bacteroides* or supposedly uncultured members of bacteria.

2. Materials and Methods

2.1 Study site description

The tidal Colne of its flushing time 0.9 day (Robinson *et al.*, 1998) is classified as a macrotidal estuary located on the east coast of north Essex, opening to the North Sea at the river mouth on Brightlingsea, as shown in **Figure 4-1**. Its catchment area is *ca* 500 km^2, consisting mainly of fertile agricultural and horticultural lands, down the Colne River. The British oldest town, Colchester has a population of *ca.* 156,000 in mid-2001 (data from the Office for National Statistics).

The Colne estuary has been characterised by strong gradients of nitrate and ammonium along the estuary (King and Nedwell, 1987) as a result of a large inputs of total nitrogen (4 to 5 x 10^7 mol N yr^{-1}) from the riverine delivery, surface runoff from the surrounding land and effluent from sewage works. Four sampling sites were chosen in this study to cover the whole salinity transect along the estuary, ranging from a seawater dominant site (Brightlingsea) and a freshwater dominant site (East Hill Bridge), where the salt

edge may reach during some high tides. Alersford and Colchester Hythe belong to the brackish water zones, and the latter site is the most nutrified site due to its proximity to a secondary treated sewage outlet in Colchester. The sediment at all sites were clay and fine silt having a 2-4% of organic carbon content (Dong *et al.*, 2000). The surface of the estuarine sediments was exposed to air periodically 10 hrs a day during low tides, appeared a wide stretch of mud flats. Further details of the study area have been well documented elsewhere (King and Nedwell, 1987; Ogilvie *et al.*, 1997b; Dong *et al.*, 2002; Kondo *et al.*, 2004).

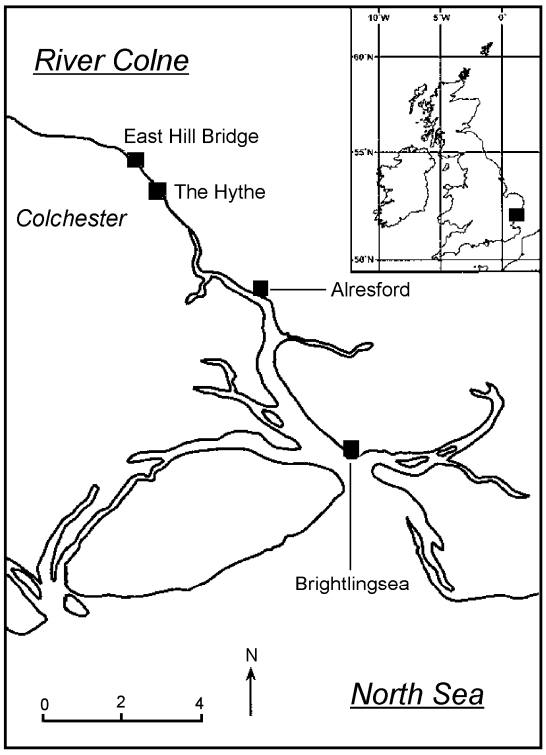


Figure 4-1 Study area, showing site locations in the Colne estuary

2.3 Sediment sampling

Bulk sediment samples were collected during a low tide from upper (0-1 cm) and deeper (3-5 cm) layers using an ethanol-washed spatula, and stored in crashed ice. The sediment samples brought back to the laboratory within 2-3 hours after sampling. Overlying water was also collected from a tidal pan formed on the nearest mud flat at the sampling point. The mud temperature was measured on site with a thermometer at a depth of *ca.* 3 cm, and the salinity of the overlying water was measured using a hand-held refract meter (Leica). All the sediment samples for investigating spatial and vertical distributions were sampled from the four sites mentioned above in October 2003, whereas test samples for optimising the PCR protocol were collected from Alresford and the Hythe in January 2001.

2.4 Nitrate and nitrite measurements

About 20 ml of pore water was obtained by centrifugation at 3,000 rpm for 15 min. Both the pore water and overlying-water were filtered through a 0.2 μm membrane filter (Sartorius). Concentration of nitrate and nitrite in water samples was measured by colourmetry using a spectrophotometer (Unicam). The procedure in details was followed by an analytical method described by Strickland and Parsons (1972). The Milli-Q (Millipore) grade water was used for chemical analysis.

2.5 DNA extraction from the sediments

Isolation of environmental DNA from 1 g (wet weight) of each sediment sample was based on a protocol of Zhou *et al.* (1996) with a few minor modifications. After the final ethanol precipitation, the DNA specimen was

resuspended in 100 µl of sterile reverse osmosis (RO) water. The extracted DNA was examined by agarose gel electrophoresis after 16S rDNA amplification, using a universal primer system (27F/1492R; Lane, 1991) to confirm the absence of PCR inhibitors in the extract, and was stored at -20°C until further analyses.

2.6 Template DNA of type cultures

Genomic DNA was extracted from an overnight culture of fermentative bacteria incubated at 30°C, according to the phenol-chloroform extraction method described by Ausubel *et al.* (1987). Model bacterial strains and sediment samples as listed in **Table 4-1** were used for extracting DNA as a template for PCR amplification. Luria-Bertani medium (10g of tryptone, 10g of yeast extract and 5g of NaCl in 1000 ml of RO water) was used for the maintenance of type cultures. *Escherichia coli* AB2463, *Klebsiella* sp. and *Serratia* sp. (originally isolated and identified with the API 20NE strips by DL Lloyd, 1999) were lab stock cultures at Essex. DNA of *Desulfovibrio desulfuricans* (DSM1926) was provided by Dr R Kondo, Fukui, Japan. Cell pellets of other sulphate-reducing bacteria were gifts from Dr H Sass, Oldenburg, Germany.

2.7 Isolates from the sediments

Fermentative bacteria were isolated from agar plates on which 0.1 ml of each dilution (10^5-10^7) of sediments was spread on a modified 2216E medium (10g of peptone and 5g of yeast extract in 1000 ml of water) and incubated at 25°C for four weeks. Salinity and pH of the media were roughly adjusted to those of the sampling locations, namely full-strength of seawater (pH 7.6) for

Table 4-1 Comparison of the *nrfA* gene detection between two primer systems

Strain	6F/6R primers[f]	F1/R1 primers[g]
Escherichia coli, AB24638 [a]	+++	+++
Enterobacter aerogenes, IAM12348	++	+
Enterobacter cloacae, IAM12349	++	+
Klebsiella pnemoniae, IAM14200	+	−
Klebsiella oxytoca, IAM14201	+	−
Serratia marcescens, IAM12142	+	−
Klebsiella sp.[a]	+	−
Serratia sp. [a]	++	−
Aeromonas encheleia [b]	+	++
Pectobacterium carotovorum [b]	+	−
Desulfovibrio desulfuricans DSM1926[c]	+	++
Desulfovibrio vulgaris [d]	+	−
Desulfovibrio saprovorans [d]	−	−
Desulfobulbus propionicus [d]	+	−
Sediment DNA, Alresford, Jan 2001	+	+
Sediment DNA, the Hythe, Jan 2001	−	+
Sediment DNA, Brightlingsea, Oct 2003	−	+
Sediment DNA, East Hill Bridge, Oct 2003	++	+++
Bacillus subtilis, IAM12118 [e]	−	−

a, Lab stocks at Essex; *b*, environmental isolates in this study; *c*, gift from Dr Kondo; *d*, gifts from Dr Sass; *e*, negative control; +++, strongly positive; ++, positive; +, weakly positive
f, semi-nested PCR primer set developed in this study; *g*, touchdown PCR primer set (Mohan et al, 2004).

Brightlingsea, 1/2-strength of seawater (pH 7.2) for Alresford and the Hythe, and tap water (pH 7.0) for East Hill Bridge. Production of acids (and gas) from glucose via fermentation was confirmed after three days' incubation at 30°C in a Hugh-Leifson's semi-solid deep agar tube (Barrow and Feltham, 1993). Nitrate and nitrite reduction of the isolates were examined by adding Griess' reagent to detect nitrite formed, later supplemented with zinc powder to detect the nitrate remained (Colwell *et al.*, 1975) after seven days' incubation at 25°C in each set of 2216E-based nitrate (0.1% $NaNO_3$) and nitrite (0.06% $NaNO_2$) broth. The isolates capable of both nitrite reduction and glucose fermentation were supposed to be putative nitrate ammonifying bacteria. Genomic DNA from these isolates was used as templates. The isolates were identified based on a Fasta3 search of EMBL/GenBank/DDBJ databases after amplifying and sequencing 16S rDNA for the domain *Bacteria* with the 27F/1492R primer pair and the 518R primer, respectively (Lane, 1991).

2.8 Design of PCR primers

Primer pairs (6F/6R and 4F/4R) were designed based on the existing partial *nrfA* sequences and complete sequences including the *nrfA* fragment in an open reading frame (ORF), which were collected from the databases. At least six *nrfA* sequences retrieved from *Escherichia coli* (AE005640, X72298), *Salmonella thphimurium* (AE008900), *Salmonella typhi* (AL627282), *Haemophilus influenzae* (U32787), *Pasturella multicoda* (AE006035), were available at the time of the primer development. Two pairs of forward and reverse primers were manually designed based on guidelines (Alkami Biosystems, 1999) and the Clustal W (Thompson *et al.*, 1994) multiple DNA sequence alignment. Two

conserved regions of the *nrfA* sequences, consisting of one based on six sequences (AE008900, AL627282, AE005640, X72298, U32787, AE006035) and the other based on four sequences (AE005640, X72298, AE008900, AL627282) were respectively chosen as the target of designing two primer pairs, 4F/4R and 6F/6R, respectively. Another primer set (F1/R1) developed by Mohan *et al.* (2004) was also employed after the PCR condition was optimised in this study. Sequences of the *nrfA* primers used in this study are listed in **Table 4-2**.

Table 4-2 Sequences of the *nrfA* primers used in this study

Primer	Sequence (5' to 3')	PCR method
6F [a]	GAY TGC CAY ATG CCR AAA GT	Semi-nested, 1st/2nd
6R [a]	GCB KCT TTY GCT TCR AAG TG	Semi-nested, 2nd
4F [a]	TTA GTC CAG TCA GAG AAG GC	no use
4R [a]	GCA TCC GCS GCT TTA TCC AT	Semi-nested, 1st
F1 [b]	GCN TGY TGG WSN TGY AA	Touchdown
R1 [b]	TWN GGC ATR TGR CAR TC	Touchdown

F: forward primer, R: reverse primer; *a*: this study, *b*: Mohan *et al.* (2004)

Base Codes: K=T/G, R=A/G, S=C/G, W=A/T, Y=C/T, B=T/C/G, N=A/C/T/G.

2.9 PCR amplification and purification

A semi-nested PCR was adopted for the first-round PCR using the 6F/4R primer set and for the second-round PCR using the 6F/6R primer set (final expected size of the *nrfA* fragment was 222 bp). A 50 μl of reaction mixture

for the PCR contained 1x PCR buffer, 200 μM dNTP mix (Roche), 2.5 mM $MgCl_2$, 1x Q-solution (only for the second-round PCR), 0.2 μM of each primer, 2.5 units of *Taq* polymerase (Qiagen) and <100 ng template DNA. The PCR amplification was performed using a programmable thermocycler, Gene Amp 9700 (Applied Biosystems). The programmes consisted of the following conditions: the initial denaturation at 94°C for 2 min, 10 cycles of 94°C for the first-round PCR (30 cycles for the second-round PCR) for 1 min, followed with an annealing temperature at 46.5°C for the first-round PCR (at 54.5°C for the second-round PCR) for 1 min and 72°C for 1 min, with the final extension step at 72°C for 10 min.

Touchdown PCR was used to amplify the *nrfA* region (expected size, 505 bp) using the1F/1R primer pair. A 50 μl of reaction mixture contained 1x PCR buffer, 200 μM dNTP mix (Roche), 7.5 mM $MgCl_2$, 1x Q-solution, 0.2 μM of each primer, 2.5 units of *Taq* polymerase (Qiagen) and <100 ng template DNA. The cycle programme was as follows: the initial denaturation at 94°C for 4 min, 30 cycles of 94°C for 1 min, an annealing temperature starting at 60°C decreased by 0.5°C per cycle for 1 min, 72°C for 1 min 30 sec, followed by additional 5 cycles of 94°C for 30 sec, 45°C for 30 sec, 72°C for 1 min, with the final extension step at 72°C for 7 min.

PCR products were separated by gel electrophoresis on agarose gel (2% for the 6F/6R primer set and 1% for the 6F/4R and 1F/1R primer sets) in TAE buffer (pH 8.0), consisting of 0.04M Tris-acetate and 0.001M EDTA (Ausubel *et al.*, 1987). A mixture of a PCR product and a loading dye solution (Type III, 1 μl) was run in the TAE buffer with a molecular marker (1kb, Life Technologies)

at 100 V for 45-90 min. After the electrophoresis, the gel was stained in an ethidium bromide (EtBr) solution (*ca* 1 µl/ml) for 15 min to visualise DNA bands using a Gel Doc Recorder (Bio-Rad Laboratories). The PCR products were purified using a spin column Quiaquick (Quiagen). Alternatively, Quick Clean (Bioline) was also used, in particular, for concentrating the PCR product up to three times prior to cloning.

2.10 Cloning of the target *nrfA* fragments

Fresh PCR products were cloned using a TA Cloning Kit (Invitrogen) supplied with a vector (pCR4-TOPO; 3,957bp) and TOP10 One Shot Chemically Competent *E. coli* cells, according to the manufacturer's instructions. White colonies were randomly isolated from Luria-Bertani agar plates containing ampicillin (Sigma, 50 g/ml) and X-Gal (Helena, 40 µl/plate) after the incubation at 37^{o}C for 1-2 days. The recombinant plasmids containing the *nrfA* were amplified by "toothpick" PCR using the T3/T7 primers. A 50 µl of reaction mixture contained 1x PCR buffer, 200 µM dNTP mix (Roche), 5 µ of α-casein solution (Sigma, 1 mg/ml), 0.1 µM of each primer, 2.5 units of *Taq* polymerase (Qiagen) and 1 µl of cell suspension as a template DNA. The cycling programme were as follows: an extended initial denaturation at 94^{o}C for 10 min to digest the carried bacterial cells, followed by 30 cycles of 94^{o}C for 1 min, an annealing temperature at 50^{o}C for 1 min, and 72^{o}C for 2 min, with the final extension at 72^{o}C for 10 min. The correct inserts of expected sizes were checked by electrophoresis, and were purified with a Qiaquick spin column.

2.11 Sequencing and diversity analysis

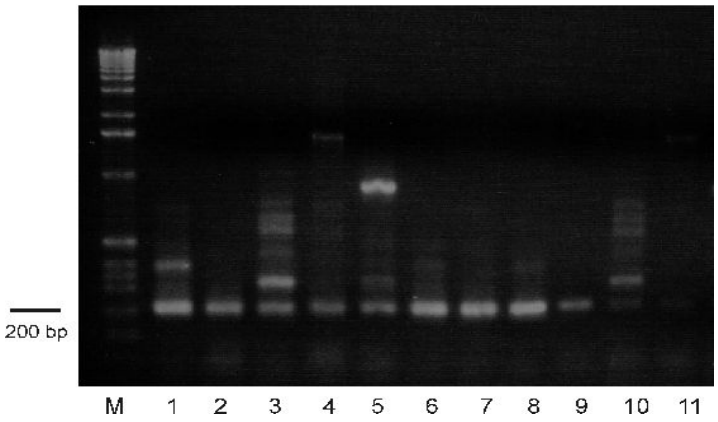
DNA sequences of the cloned PCR amplicons were determined with a Big

Dye Terminator Cycle Sequencing Kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems), according to the manufacturer's recommendations. The T3 and/or T7 primer was employed for cycle sequencing. The sequence data were edited using a Chromas2 programme, and were subjected to a similarity search engine to extract the best matching alignment from the EMBL database using a Fasta3 programme. Similarity values based on multiple sequence alignments were compiled using a Clustal W programme. Phylogenetic trees were generated using the neighbour-joining method as implemented with a TreeView programme (Page, 1996).

3. Results and discussion

3.1 Optimisation of the PCR protocols

Optimum conditions for PCR amplification of the *nrfA* gene were evaluated among four combinations of the two primer pairs, namely, F6/R6, F6/R4, F4/R6, and F4/R4. Of these pairs, the best result was obtained by the F6/R4 set for the first-round PCR and the F6/R6 set for the second-round of a semi-nested PCR. The best cycle numbers were 10 cycles for the first reaction and 30 cycles for the second reaction. The addition of Q-solution (1x) for the second-round reaction was effective in increasing the final yields and reducing unspecific products. The addition of α-casein (5 μl/ 50 μl of the PCR mixture) was sometimes useful for environmental DNA but not for culture-derived DNA. Increase of the final Mg^{2+} concentration in the PCR mixture up to 5.5 mM was not effective (data not shown). As a result, the most optimised conditions for the semi-nested PCR were as described in the methodology section. As



The second round PCR products of the expected size (200 bp) were visualised on 2% agarose gel stained with ethidium bromide. Lanes: M, 1 kb DNA ladder (Life Technologies); 1, *Escherichia coli* (AB2463); 2, *Klebsiella* sp.; 3, *Serratia* sp.; 4, *Aeromonas encheleia* (Hw25/NA10); 5, *Pectobacterium carotovorum* (Bw10/NA12); 6, *Enterobacter aerogenes* IAM12348; 7, *Enterobacter cloacae* IAM12349; 8, East Hill Bridge sediment; 9, Alresford sediment; 10, *Desulfovibrio desulfuricans* DSM1926; 11, negative control.

Figure 4-2 The *nrfA* gene, amplified by a semi-nested protocol using the 6F/4R and 6F/6R primer pairs.

shown in **Figure 4-2,** the final yield was not always satisfactory for cloning of the environmental DNA unless the products were concentrated. Although there remained some unspecific bands in the PCR products from culture-derived DNA, these multiple bands could be reduced by adjusting annealing temperature suitable for each species. This protocol worked for a wider range of templates derived from type strains and environmental isolates as summarised in **Table 4-1**. The two-step PCR procedure may be reasonable for rare samples but not for further quantitative PCR. However, this primer set would be still useful to fulfill the gap between the sequences from uncultured and cultured bacteria of environmental origins.

Another *nrfA* primer system (F1/R1) developed by Mohan *et al.* (2004) was also optimised for PCR amplification of the environmental DNA extracted from sediment samples. The total number of thermal cycling for a touchdown PCR was reduced from a total of 60 cycles to the initial 30 cycles, followed by 5 more cycles at the fixed annealing temperature to improve the yield and avoid the increase of unspecific background. Double concentration of the primers from 0.1 μM to 0.2 μM in the PCR mixture increased the yield. The addition of Q-solution (1x) was constantly effective to increase the yield, particularly for the environmental DNA. The amplified products were concentrated up to three-fold using a Quick Clean (Bioline) to improve the cloning efficiency. This primer set was not likely to cover a wider range of taxa (**Table 4-1**), but proved to have an advantage in obtaining a longer *nrfA* sequence (*ca* 500 bp) with a single step of PCR, leading to a quantitative analysis in the future. Therefore, this primer system and the protocol optimised in this study were applied to the following field-oriented investigation.

3.2 Environmental gradients of the estuary

Salinity can be regarded as a good indicator of mixing seawater with freshwater along the tidal channel of an estuary. Based on monthly observations throughout 2002, salinity in the upper estuary (the Hythe) was very fluctuating between 1-16‰ (mean 7‰), while that in the lower estuary (Brightlingsea) was rather stable around 33‰ (almost seawater). Freshwater dominance was apparent at the East Hill Bridge in the uppermost end of the tidal zone just in front of a weir. **Figure 4-3** shows a profile of water quality measured on the day of sediment sample collection, exhibiting a clear gradient

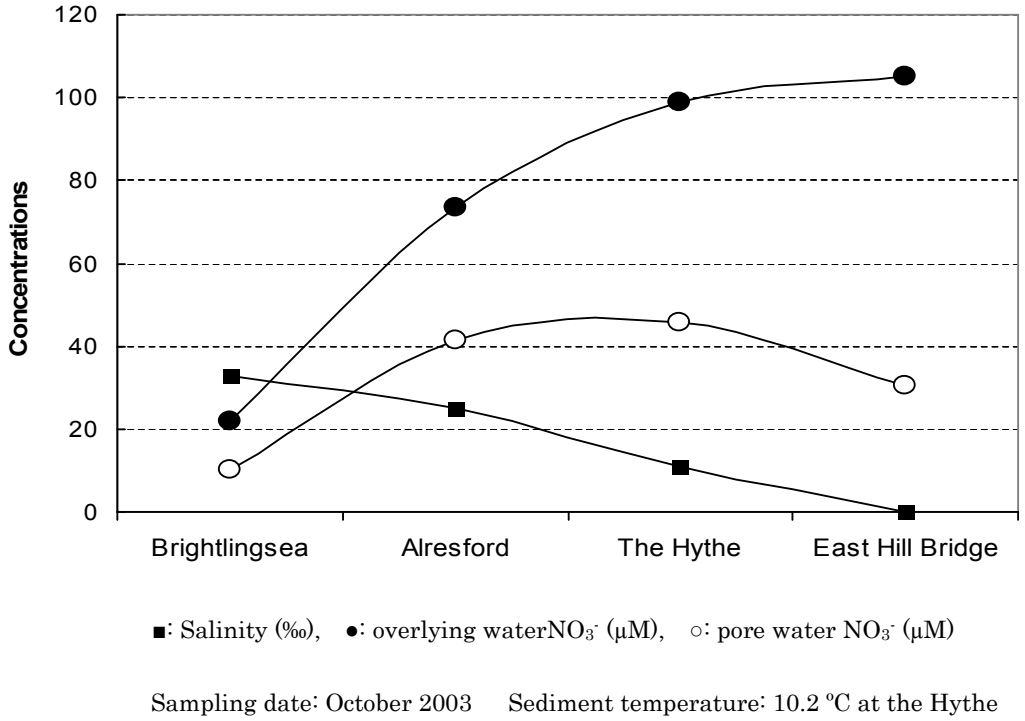


Figure 4-3 Environmental gradients along the Colne estuary

of salinity up to the Colne estuary was observed.

The decreasing gradient of nitrate content down the estuary was observed both in overlying water and in pore water of the sediment along the estuary. Based on the monthly observations in 2002, the annual mean concentrations of nitrate in the overlying water both at Alresford and the Hythe were higher (65.8 μM and 66.9 μM, respectively) than that at Brightlingsea (19.7 μM). Nitrate concentrations in the pore water were generally lower than those in the overlying water, which can be directly influenced by external loading of nitrate. However, nitrate concentrations in the water of the Colne estuary widely varies from time to time, directly influenced by surrounding conditions. According to a field survey (EC Agedah, unpublished) carried out in 2002-2003, over 1 mM

of nitrate concentration in the surface water was recorded at the maximum value just down the Hythe site along the main tidal course.

Another field survey in 2000 (I Rusmana, PhD thesis, Essex, 2003) suggested that nitrate tended to be abundant in the upper (0-3 cm) layers (15-18 μM at the Hythe and 3-7 μM at Brightlingsea), while nitrate was generally scare in the deeper (3-5 cm) layers (< 6 μM at the Hythe and 0-4 μM at Brightlingsea). Nitrite was scarce, usually less than 2 μM. In contrast, ammonium gradually increased with the sediment depth (150-250 μM at a 3-5 cm deep in both sites). There seemed to be a critical interface at a 3 cm deep in terms of anoxic and anaerobic zones influenced by the downward diffusion of nitrate. Thus, the deeper sediments at 3-5 cm deep were compared with those from the surface sediment at 0-1 cm throughout this study.

3.3 Vertical distribution patterns in the sediments

The structure of nitrate ammonifying community at different sediment depths was analysed based on the *nrfA* partial sequences. Three sites, the East Hill Bridge, the Hythe and Brightlingsea were chosen to represent freshwater, brackishwater and seawater habitats, respectively. As shown in **Figures 4-4 and 4-5**, two distinct communities were clearly grouped according to the depth of sediments at the East Hill Bridge and Brightlingsea sites, respectively. However, the *nrfA* clones retrieved from the Hythe sediment did not exhibit this tendency, being the clones from the different horizons mixed together and scattered within a single cluster (data not shown).

The similar tendency as the East Hill Bridge was also found in Brightlingsea, where only three of the *nrfA* clones (Bd66, Bd70 and Bd71) from the deeper

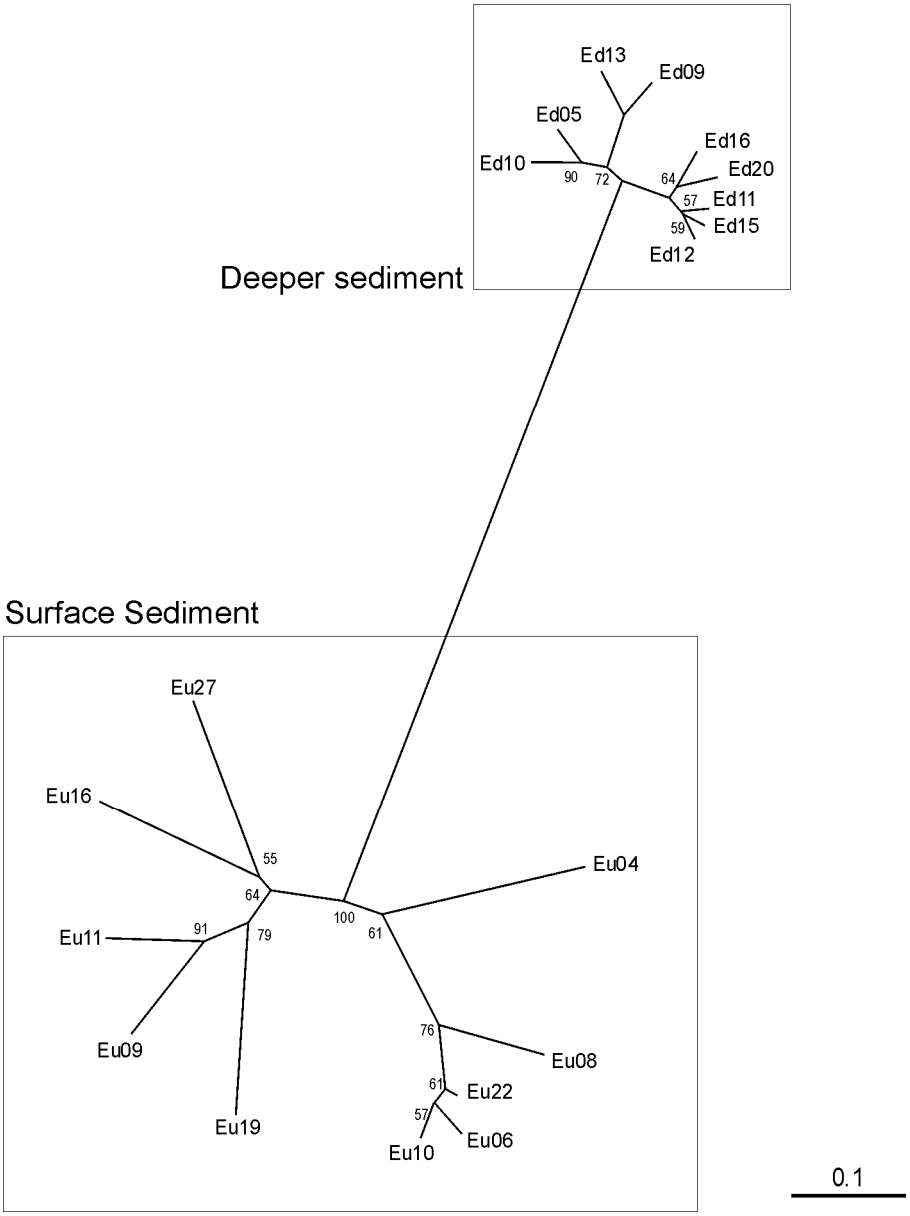


Figure 4-4 Vertical distribution of the *nrfA* clones from freshwater sediment at the East Hill Bridge.

Eu: the *nrfA* clones from the surface sediment, Ed: the *nrfA* clones from the deeper sediment. The bar indicates 10% nucleotide sequence divergence. Numbers near the nodes represent the percentages of 1,000 bootstrap repetitions. Confidence limits of less than 50% are not shown.

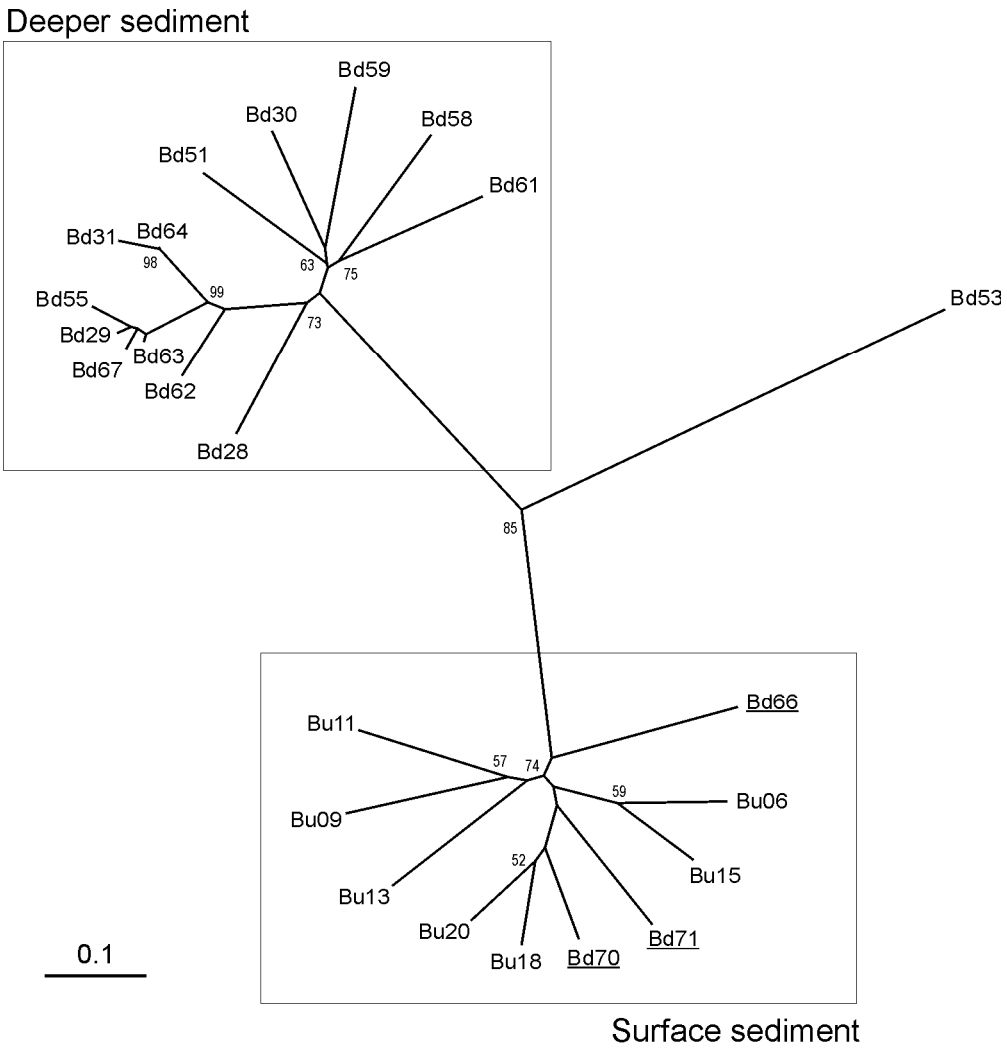


Figure 4-5 Vertical distribution of the *nrfA* clones from coastal sediment at Brightlingsea.

Bu: the *nrfA* clones from the surface sediment, Bd: the *nrfA* clones from the deeper sediment. Only three clones from the deeper sediment (marked with underbars) were mixed in the surface cluster. The bar indicates 10% nucleotide sequence divergence. Numbers near the nodes represent the percentages of 1,000 bootstrap repetitions. Confidence limits of less than 50% are not shown.

layer were mixed with those from the surface layer. However, none of the *nrfA* clones derived from the surface layer were found in the deeper layer.

This suggests that the *nrfA* populations in the lower estuary may occasionally migrate from the deeper to upper horizons, presumably by mechanical and/or biological mixing (bioturbation).

On closer examination of the tree structure (e.g. the length of each branch) of the East Hill Bridge, the *nrfA* clones from the deeper layer of the sediment formed a more homogenous community than those from the surface layer of the sediment. This may be due to highly isolated and reduced conditions kept in the deeper sediment scarcely influenced by the overlying water and other environmental factors. This result meets the appearance of the sediment core profile, which was clearly divided into oxic and anaerobic zones indicated by the colour (grey and black, respectively) at the East Hill Bridge site. This boundary was not so clear at other three sites. Although the East Hill Bridge site is predominantly freshwater but a salt wedge occasionally penetrates as a result of the interplay of tidal and river flows. This periodic input of sulphate of a seawater origin is large enough to sustain high rate of sulphate reduction to develop the blacken horizons even in freshwater sediments (Capone and Kiene, 1988). Besides, metabolically versatile sulphate reducing populations (the *Desulfobulbaceae*) in the sediment of East Hill Bridge may be involved in anaerobic digestion of rich organic matter deeply buried (Kondo *et al.*, 2004). The predominance of these strictly anaerobic microbes leads to the establishment of more stable and self-perpetuating consortia in the deeper horizons. Hence, the restricted community developed in the deep sediment suggests that the microbes are not mobile and utilise limited nutrients. As a result of their survival and reproduction within

the isolated habitat, the molecular diversity of the *nrfA* community could become lower and lower, forming an extremely conserved gene pool consisting of only closely related gene sequences, presumably originated from the same populations. Freshwater sediments can also be disturbed by flushing after heavy rain events, but the opportunities of disturbance were not as frequent as estuarine and marine sediments subjected to daily tidal rise and fall.

In contrast, the bacterial community inhabiting the surface sediment can frequently be influenced by other environmental factors; not only mechanical disturbance but also external nutrient supply and microbial colonisation from some other sources. Novitsky (1990) demonstrated that particle-associated bacteria transported from the water column were a major source of the surface sediment. One possible reason for homogeneous composition of the *nrfA* clones at the Hythe could be the mobility of sediment particles easily suspended in the middle reach of the tidal channel, which is referred to as "the turbidity maximum". Thus, particular emphasis was placed on the surface sediments as more sensitive habitats when investigating a longitudinal profile of the *nrfA* gene distribution along the Colne estuary.

3.4 Spatial distribution patterns in the sediments

The distribution pattern of the *nrfA* gene cloned from the surface sediments (0-1 cm) along the salinity gradients of the Colne estuary was investigated. As shown in **Figure 4-6,** the *nrfA* clones distributed in the four sites covering a wide range of salinity formed two distinct clusters corresponding to their geographical locations. The clones collected from brackish water (Alresford and the Hythe) habitats were clearly separated from the other cluster consisting

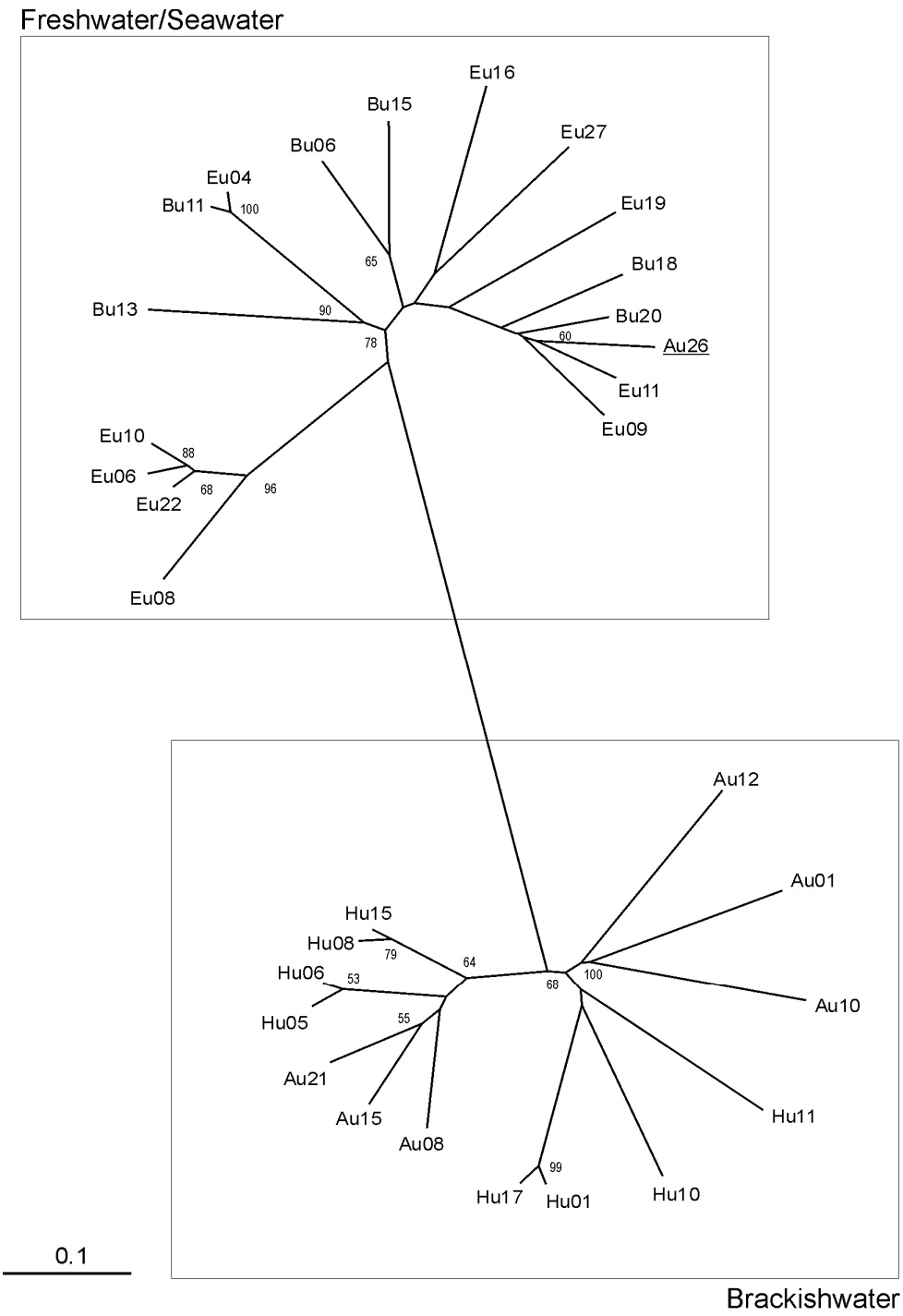


Figure 4-6 Spatial distribution of the *nrfA* clones in the surface sediments

The *nrfA* clones from East Hill Bridge (Eu), the Hythe (Hu), Alresford (Au) and Brightlingsea (Bu). The bar indicates 10% nucleotide sequence divergence. Numbers near the nodes represent the percentages of 1,000 bootstrap repetitions. Confidence limits of less than 50% are not shown.

of freshwater (East Hill) and seawater (Brightlingsea) habitats. Only one clone (Au26) from a brackish water environment was exceptionally found in the freshwater/seawater cluster. This result suggests that salinity *per se* was not likely to affect the selection of the *nrfA* bacterial populations.

One supportive fact was fermentative nitrate ammonifiers predominated in anaerobic enrichment cultures irrelevant to NaCl concentrations from 0 to 0.4 M, simulating freshwater to seawater, respectively (Dunn *et al.*, 1980). The other fact was that the nitrate ammonifying activity can be equally detected in aquatic sediments with a wide range of salinity. These facts suggest that the nitrate ammonifiers widely occupy the same ecological niche, ranging from freshwater to marine habitats. Then, what factors divided the *nrfA* clones into two distinct clusters, namely, the brackishwater group and the freshwater/seawater mixed group?

Phylogenetic structure of estuarine bacterial community along the salinity gradient has been known to be divided into three geographical location-specific groups. For example, Crump *et al.* (1999) investigated the longitudinal succession of bacterial community in the water along the Columbia estuary, standing out a contrast in their phylogenetic composition between them of different origins such as riverine, estuarine and marine samples. Similar results were reported from different estuaries, in which phylogenetic community was well separated according to the salinity gradient (Bouvier and Del Giorgio, 2002; Del Giorgio and Bouvier, 2002). Furthermore, Selje and Simon (2003) pointed out that bacterial communities were well separated according to the salinity-related locations rather than seasonal changes.

Based on these findings, salinity seemed to be more definitive pressure to induce the spatial succession of the whole bacterial community along an estuarine system.

However, this view on biogeography of the bacterial community in an estuarine environment has been introduced by the universal marker of phylogenetic genes. Therefore, it does not really reflect their metabolic functions. In contrast with the past studies aimed at the phylogenetic analysis, this study was focused on the specific community responsible for nitrate ammonification. Since the *nrfA* sequences have been so far extracted from various sources (bioreactors and estuarine sediments) as well as from laboratory cultures beyond the phylogenetic groups (α-, γ-, δ-, ε-proteobacteria and the *Bacteroides*-related subdivisions), it is quite reasonable to detect the similar *nrfA* sequences of different phylogenetic origins in the environmental samples (e.g., one from the *Enterobacteriaceae* and the other from sulphate-reducing bacteria). This approach makes it possible to detect the functional marker *in situ* but may reduce its analytical resolution into the community structure. As a result, the *nrfA*-based grouping was not able to distinguish the freshwater from seawater clones but successful in extracting the estuarine clones from the others.

As discussed in the previous section, stability of the environment seemed to affect the distribution pattern of the specific functional genes, evolving habitat segregation between estuarine and the other adjacent zones. Although the East Hill Bridge and Brightlingsea sites are naturally in strong contrast in terms of salinity, and besides being these locations geographically distant from each

other, both habitats are thought to be more stable as compared with the estuarine zones, which experience greater and frequent environmental changes such as salinity and turbidity. Capone and Kiene (1988) pointed out that estuaries were highly variable and dynamic systems and the sediments *per se* were often subjected to routine resuspension and scouring by tidal movements. In this sense, Alresford and the Hythe sites located in the tidal water course may be different from other two sites whose conditions are much more stable, apparently predominated by freshwater and seawater, respectively.

Capone and Kiene (1988) also suggested the difference in relative importance of anaerobic respiratory modes in various aquatic sediments, namely, sulphate reduction in marine habitats and methanogenesis in freshwater habitats. According to their model, nitrate respiration observed all kinds of sediments, particularly with highest rates found in mid-salinities, receiving agricultural and sewage discharges. Nitrate ammonification is a terminal metabolic pathway of nitrate respiration. Therefore, the habitat segregations of the *nrfA* clones were observed between brackish habitats and seawater/freshwater habitats, which may be reflected by environmental variability.

As shown in **Figure 7**, the *nrfA* community structure in the deeper layers was relatively homogenous as compared with that in the surface layers (**Figure 6**). In particular, the *nrfA* clones from freshwater habitat tended to form a distinct cluster, whereas a few of the clones from the deep marine sediment (Bd53, Bd66, Bd70 and Bd71) were quite divergent from the others and affiliated with the *nrfA* sequences retrieved from versatile anaerobes such as

sulphur-reducing nitrate ammonifier *Sulfurospirillum* deleyianum (Eisenmann *et al.*, 1995) and iron-reducing nitrate ammonifier *Geobacter sulfurreducens* (Lonergan *et al.*, 1996). These microbes seem to be favoured anaerobic marine sediments full of iron sulphide, involved in sulphur and iron cycles coupled with nitrate ammonification.

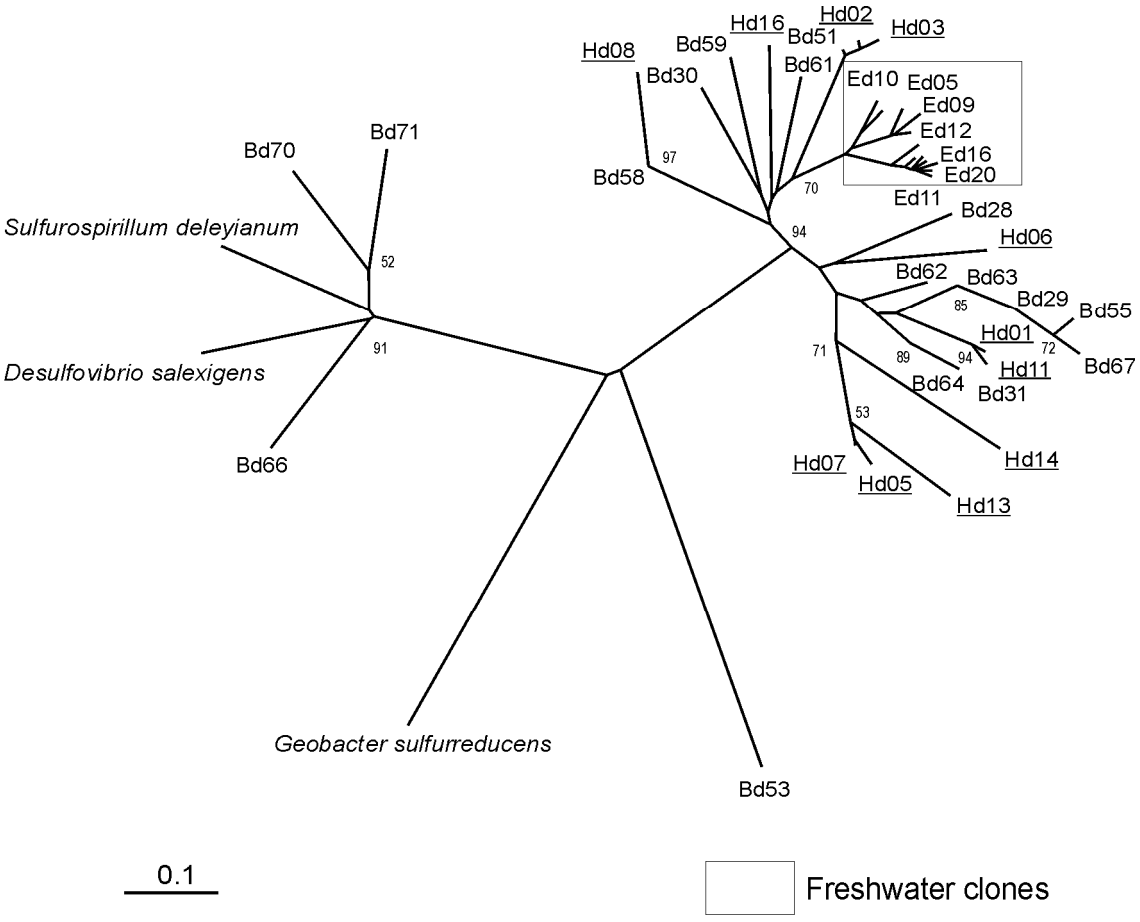


Figure 4-7 Spatial distribution of the *nrfA* clones in the deeper sediments

The *nrfA* clones from East Hill Bridge (Ed), the Hythe (Hd) and Brightlingsea (Bd). Three reference sequences from *Sulfurospillum deleyianum* (AJ133037), *Desulfovibrio salexigens* (AJ697977) and *Geobacter sulfurreducens* (AE017180) were included. The *nrfA* clones of the estuarine origin were marked with underbars, while those of the freshwater origin were marked in a box. The bar indicates 10% nucleotide sequence divergence. Numbers near the nodes represent the percentages of 1,000 bootstrap repetitions. Confidence limits of less than 50% are not shown.

However, the similarity values of the *nrfA* sequences from the environment were usually 60-70%, being smaller than those of denitrifying genes such as *nirS*, *nirK* and *nosZ* (70-80% identity, detailed data not shown) found in the same study sites. Most of the cloned *nrfA* sequences from the environmental samples were phylogenetically distant from any cultured species because of limited numbers of the *nrfA* sequences currently available from the existing databases.

3.5 Diversity of the *nrfA* sequences in the sediments

An overview of the diversity of the *nrfA* sequences in the Colne sediments was also examined. A total of 63 sequences retrieved from the clones found in the sediments were compare with those from known strains and environmental isolates. **Figure 4-8** shows a dendrogram, suggesting the relationship of partial nucleotide sequences form the *nrfA* clones and the other reference sequences obtained from the databases. Two environmental isolates were identified as *Aeromonas enchleia* (99.6% identify of a 16S rDNA basis) and *Pectobacterium carotovorum* (99.2% identity) in this study, and their *nrfA* sequences were also included in the analysis.

Three nucleotide sequence clusters were roughly grouped: One cluster (B) belonged to the known group as nitrate ammonifiers with the *nrfA* sequences, comprised of fermentative bacteria (*Escherichia coli*), sulphate-reducing bacteria (*Desulfovibrio desulfuricans*) and the others. Most of the *nrfA* clones from the Colne sediments were closely related to the *nrfA* sequences from *Shewanella*, *Porphyromonas*, and *Sulfurospirillum*, while traditionally known nitrate ammonifiers tended to form a distinct subgroup but divergent from the

environmental *nrfA* clones as well as those from wastewater treatment reactors (AJ38-AJ48; Mohan *et al.*, 2004). Two strains, *Aeromonas enchleia* and *Pectobacterium carotovorum*, were originally isolated from the sediments in the Hythe and Brightlingsea, respectively. However, neither of these isolates formed the same subgroup with the environmental *nrfA* clones, suggesting a methodological difference between the cultural-depending and cloning approaches. Almost all of the *nrfA* sequences of bioreactor origins (AJ38-AJ46) formed a subgroup with *Porphyromonas*, excepting for AJ48. Most of the bioreactor-derived *nrfA* sequences were affiliated with those from the freshwater sediment. This result may be reasonable; as the bioreactors were usually applied to wastewater of a freshwater basis, suggesting the similar ecological niche to be functionally shared by sediment- and bioreactor-origin microorganisms.

The second group of the *nrfA* clones formed a distinct cluster (C) which was closely related to those from the *Bacteroides* group; these still divergent from the major *nrfA* cluster (B). In particular, the cluster C included the *nrfA* clones retrieved from the anaerobic deeper sediments, which may reflect their possible adaptation to the anaerobic condition. The *nrfA* sequences grouped in the third cluster (A) were quite divergent from the known *nrfA* sequences, which suggested the existence of unknown group of the bacterial population responsible for nitrate ammonification *in situ*. None of the *nrfA* clones from the freshwater sediment were found in the cluster A, while most of the *Bacteroides*-related *nrfA* sequences were affiliated with those from the freshwater sediment.

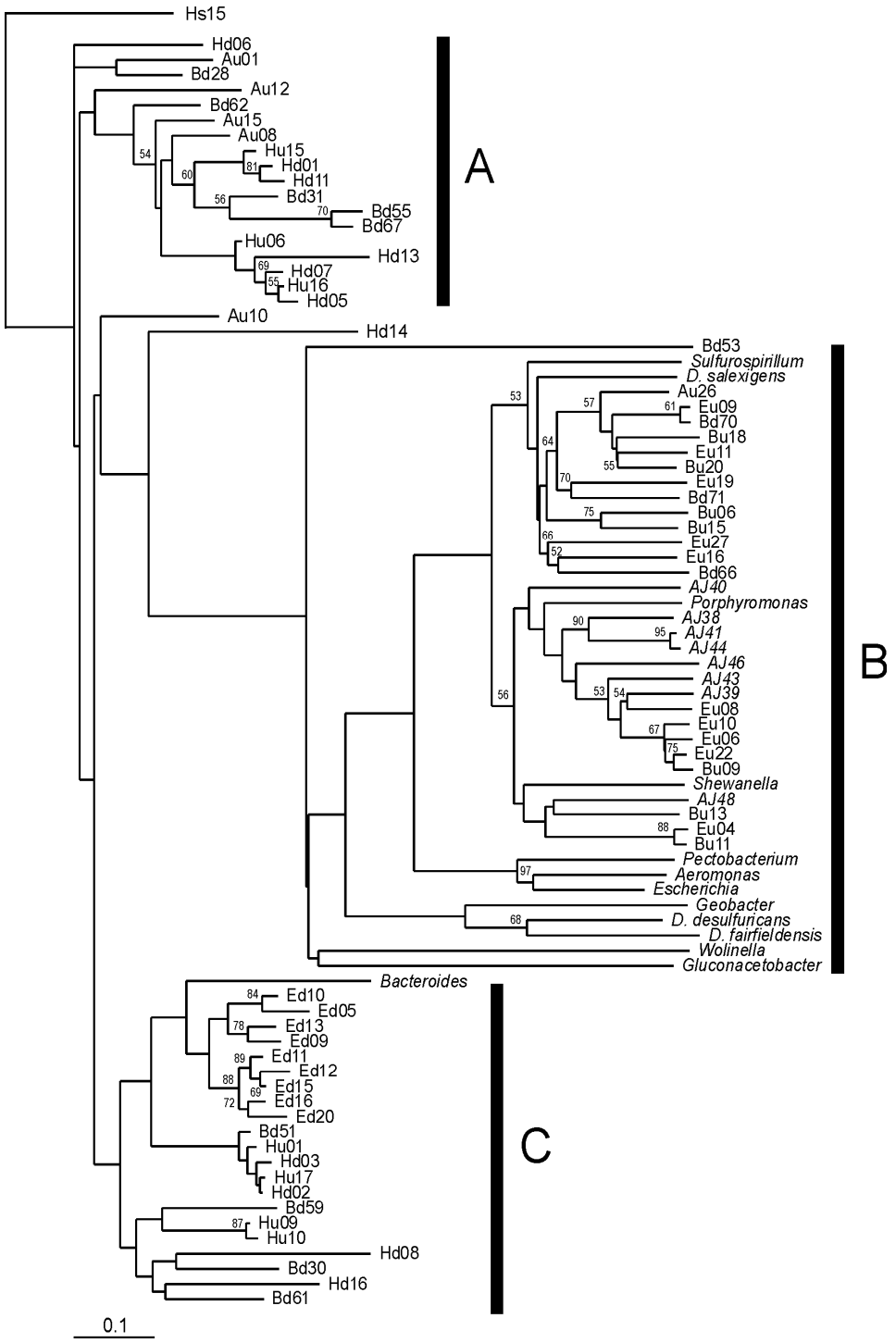


Figure 4-8 Dendrogram showing the diversity of the *nrfA* partial nucleotide sequences from the clones, nitrate ammonifying isolates from the Colne estuary, with reference sequences from the databases

Sulfurospirillum deleyianum (AJ133037), *Desulfovibrio salexigens* (AJ697977), *Porphyromonas* (formerly, *Bacteroides*) *gingivalis* (AE017178), *Shewanella oneidensis* (AE01528), *Pectobacterium carotovorum* (Bw10/NA12), *Aeromonas enchdeia* (Hw25/NA10), *Escherichia coli* (X72298), *Geobacter sulfurreducens* (AE017180), *Desulfovibrio desulfuricans* (AJ316232), *Desulfovibrio fairfieldensis* (AJ697978), *Wolinella succinogenes* (BX571659), *Gluconacetobacter diazotrophicus* (AF494454), *Bacteroides thetaiotaomicron* (AE016931). The *nrfA* partial sequences from *AJ38* to *AJ48* were originated from anaerobic reactors for wastewater treatment, whose original accession numbers are from AJ697838 to AJ697848. The codes for the *nrfA* sequences in this study are expressed as a combination of each sampling site and the sampling layer, e.g., H for the Hythe and Hu from the surface and Hd from the deeper layers. Hs15: the *nirK* partial sequences, retrieved from the Hythe, encoding another type of nitrite reductases as the outgroup. The bar indicates 10% nucleotide sequence divergence. Numbers near the branches represent the percentages of 1,000 bootstrap repetitions. Confidence limits of less than 50% are not shown.

Although the present result partly supported the previous view on the contribution of fermentative bacteria to nitrate ammonification, it also suggested the majority of the actual agents could be other metabolically versatile but less described groups such as *Shewanella*, *Sulfurospirillum* and *Bacteroides*. Widespread distribution of these *nrfA* sequences found in this study could be a result of their adaptation to nitrate and organics rich habitats. The nitrate ammonifying populations in the sediment have been so far treated as a minor component but ecologically important not only as a consumer of nitrite but also as a producer of ammonium. Further studies on the *nrfA* gene in the environment are needed to discuss the ecological relationships such as competition of the nitrate ammonifying populations with the denitrifying populations for nitrite and possible commensalisms with the anammox (anaerobic ammonium oxidation) microbes that require both ammonium and nitrite, which fulfils another missing link of the biological nitrogen cycle (Devol, 2003). Although the anammox process was found in wastewater treatment

reactors (Mulder *et al.*, 1995), this unique pathway was also confirmed in marine (Thamdrup and Dalsgaard, 2002) and estuarine sediments (Trimmer *et al.*, 2003). Nitrate ammonifiers could play an important role as a supplier of ammonium to the anammox microbes, two long overlooked short circuits to be linked up to fulfill the actual nitrogen cycle.

4. Concluding remarks

The *nrfA*-specific primers were successfully optimised and amplified both culture-derived and environmental DNA in this study, leading to the *nrfA* clone library construction. A computer-aided analysis of the *nrfA* clones brought clear-cut differences in the spatial and vertical distribution patterns according to their habitat characteristics. Salinity did not affect the distribution pattern of the *nrfA* clones but possible disturbance of the bottom sediment by tidal force may be more influential in dividing them into unstable estuarine and the other stable habitats. The *nrfA* clones embedded in deeper anaerobic sediments showed more homogenous distribution as compared with those in surface sediments, whose conditions tend to be more influenced by environmental factors such as oxygen and nitrate diffusion. The present results partly supported the traditional knowledge that the fermentative bacteria catalysed nitrate ammonification. However, other versatile microorganisms such as *Sulfurospillum* and *Geobacter* seemed to be engaged in this process, presumably coupled with sulphur and iron reduction in the anaerobic sediment. In addition, *Bacteroides* and other unknown members of bacteria may also be involved in nitrate ammonification..

References

Alkami Biosystems (1999) *Alkami Quick Guide for PCR* Vol. I, 78pp. Alkami Biosystems, Berkeley.

Ausubel FM, Brent R, Kingston RE, Moor DD, Seidman JG, Smith JA, Struhl K (1987) *Current Protocols in Molecular Biology*, 1600pp. John Wiley & Sons, New York.

Barrow GI, Feltham RKA (1993) *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 331pp. Cambridge University Press, Cambridge.

Bouvier TC, Del Giorgio PA (2002) Compositional changes in free-living bacterial communities along a salinity gradient in two temperate estuaries. *Limnol Oceanogr*, **47**: 453-470.

Braker G, Fesefekdt A, Witzel K-P (1998) Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Appl Environ Microbiol,* **64**: 3769-3775.

Braker G, Tiedje JM (2003) Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. *Appl Environ Microbiol*, **69**: 3476-3483.

Capone DG, Kiene RP (1988) Comparison of microbial dynamics in marine and freshwater sediments: Contrasts in anaerobic carbon catabolism. *Limnol Oceanogr*, *33*: 725-749.

Cole JA (1978) The rapid accumulation of large quantities of ammonia during nitrite reduction by *Escherichia coli*. *FEMS Microbiol Lett,* **4**: 327-329.

Cole JA, Brown CM (1980) Nitrite reduction to ammonia by fermentative bacteria: a short circuit in the biological nitrogen cycle. *FEMS Microbiol Lett*, **7**: 65-72.

Colwell RR, Sizemore RK, Carney JF, Nelson Jr JD, Picker JH, Schwarz J, Walker JD, Morita RY, Van Valkenburg, SD, Wright RT (1975) *Marine and Estuarine Microbiology Laboratory Manual*, 96pp. University Park Press, Baltimore.

Crump BC, Armbrust EV, Baross JA (1999) Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. *Appl Environ Microbiol,* **65**: 3192-3204.

Del Giorgio PA, Bouvier TC (2002) Linking the physiologic and phylogenetic successions in free-living bacterial communities along an estuarine salinity gradient. *Limnol Oceanogr*, **47**: 471-486.

Devol AH (2003) Solution to a marine mystery. *Nature* **422**: 575-576.

Dong LF, Thornton DCO, Nedwell DB, Underwood GJC (2000) Denitrification in sediments of the River Colne estuary, England. *Mar Ecol Prog Ser,* **203**: 109-122.

Dong LF, Nedwell, DB, Underwood GJC, Thornton DCO, Rusmana I (2002) Nitrousoxide formation in the Colne Estuary, England: the central role of nitrite. *Appl Environ Microbiol*, **68**: 1240-1249.

Dunn GM, Wardell JN, Herbert RA, Brown CM(1980) Enrichment, enumeration and characterisation of nitrate-reducing bacteria present in sediments of the River Tay estuary. *Pro Royal Soc Edinburgh*, **78B**: s47-s56.

Eisenmann E, Beuerle J, Sulger K, Kroneck PMH, Schmacher W (1995) Lithotrophic growth of *Sulfurspirillum deleyianum* with sulphide as electron donor coupled to respiratory reduction of nitrate to ammonia. *Arch Microbiol*, **164**: 180-185.

King D, Nedwell DB (1985) The influence of nitrate concentration upon the end-products of nitrate dissimilation by bacteria in anaerobic salt marsh sediment. *FEMS Microbiol Ecol*, **31**: 23-28.

King D, Nedwell DB (1987) The adaptation of nitrate-reducing bacterial communities in estuarine sediments in response to overlying nitrate load. *FEMS Microbiol Ecol*, **45**: 15-20.

Koike I, Hattori A (1978) Denitrification and ammonia formation in anaerobic costal sediments. *Appl Environ Microbiol*, **35**: 278-282.

Kondo R, Nedwell DB, Purdy KJ, Silva, SQ (2004) Detection and enumeration of sulphate-reducing bacteria in estuarine sediments by competitive PCR. *Geomicrobiol J*, **21**: 145-157.

Lane DJ (1991) 16S/23S rRNA sequencing. In E. Stackebrandt and M Goodfellow (eds.) *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115-175. John Wiley & Sons, Chichester.

Lonergan DJ, Jenter HL, Coates JD, Phillips EJ, Schmidt TM, Lovley DR (1996) Phylogenetic analysis of dissimilatory Fe (III)-reducing bacteria. *J Bacteriol*, **178**: 2402-2408.

Malone TC (1984) Anthropogenic nitrogen loading and assimilation capacity of the Hudson River estuarine system, USA. In: Kennedy VS, editor. *The Estuary as a Filter*, pp. 291-311, Academic Press, Orlando.

Mohan SB, Schmid M, Jetten M, Cole J (2004) Detection and widespread distribution of the *nrfA* gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. *FEMS Microbiol Ecol*, **49**: 433-443.

Mulder A, Van de Graaf AA, Robertson LA, Kuenen JG (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiol Ecol*, **16**: 177-184.

Nedwell DB (1975) Inorganic nitrogen metabolism in a eutrophicated tropical mangrove estuary. *Water Res*, **9**: 221-231.

Nedwell DB, Jickells TD, Trimmer M, Sanders R (1999) Nutrients in estuaries. *Adv Ecol Res*, **29**: 43-92.

Novitsky JA (1990) Evidence for sedimenting particles as the origin of the microbial community in a coastal marine sediment. *Mar Ecol Prog Ser*, **60**: 161-167.

Ogilvie BG, Rutter M, Nedwell DB (1997a) Selection by temperature of nitrate-reducing bacteria from estuarine sediments: species composition and competition for Nitrate. *FEMS Microbiol Ecol*, **23**: 11-22.

Ogilvie B, Nedwell DB, Harrison RM, Robinson A, Sage A (1997b) High nitrate, muddy estuaries as nitrogen sinks: the nitrogen budget of the River Colne estuary (United Kingdom), *Mar Ecol Prog Ser*, **150**: 217-228.

Owens NJP (1986) Estuarine nitrification: a naturally occurring fluidized bed reaction? *Estuar Coastal Shelf Sci*, **22**: 31-44.

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Comp Appl Biosci*, **12**: 357-358.

Robinson AD, Nedwell DB, Harrison RM, Oglvie BG (1998) Hypernutrified estuaries as sources of N_2O emission to the atmosphere: the estuary of the River Colne, Essex, UK. *Mar Ecol Prog Ser*, **164**: 59-71.

Scala DJ, Kerkhof LJ (1998) Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. *FEMS Microbiol Lett*, **162**: 61-68.

Selje N, Simon M (2003) Composition and dynamics of particle-associated and free-living bacterial communities in the Weser estuary, Germany. *Aquat Microb Eco*l, **30**: 221-237.

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

Strickland JDH, Parsons TR (1972) *A Practical Handbook of Seawater Analysis*, pp. 71-76, Fish Res Board Can, Ottawa.

Taniguchi S, Sato R, Egami F (1956) The enzymatic mechanisms of nitrate and nitrite metabolism in bacteria, In *Inorganic Nitrogen Metabolism*, ed by B Glass, pp. 87-108, Johns Hopkins University Press, Baltimore.

Thamdrup B, Dalsgaard T (2002) Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. *Appl Environ Microbiol*, **68**: 1312-1318.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Acids Res*, **22**: 4673-4680.

Tiedje JM, Sexstone AJ, Myrold DD, Robinson JA (1982) Denitrification: ecological niches, competition and survival. *Antonie van Leeuwenhoek*, **48**: 569-583.

Tiedje JM(1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium, In *Biology of Anaerobic Microorganisms*, ed by AJB Zehnder, pp.179-244, John Wiley & Sons , New York.

Trimmer M, Nicholls JC, Deflandre B (2003) Anaerobic ammonium oxidation measured in sediments along the Thames estuary, United Kingdom. *Appl Environ Microbiol*, **69**: 6447-6454.

Zhou J, Burns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. *Appl Environ Microbiol*, **62**: 316-322.

Chapter 5

Extended Summary and the future perspectives:

Ecological implications of nitrate ammonification for studying a missing link of the biogeochemical nitrogen cycle

1. Nitrate ammonification as a lost pathway

Nitrogen is abundant in the air but not directly available to most organisms. Therefore, before the invention of industrial nitrogen fixation, we had struggled against this lack of nitrogen. It was an epoch-making solution against nitrogen shortage and for making use of the inert gas for agricultural fertilizers.

Fixed nitrogen has brought us more food supplies but also, unfortunately, serious water pollution by discharging human waste, couple with urbanization. Sewage treatment widely implemented in western countries has been expected to be a promising measure against the pollution. Although it works partially for reducing organic pollutants, decomposed inorganic nitrogen acts as fertilizers leading to eutrophication in lakes and closed bays. Water blooms and red tides have caused a severe damage to the water-related industries.

When it comes to removing nitrogen from the water, we should concern about an unexpected shortcut from nitrate to ammonia, called nitrate ammonification, in which dissolved nitrogen is not always removed from the water. This pathway had been discovered by marine geochemists, but it has been ignored as a classical missing link for a long period. A few aquatic microbiologists, mainly based in Dundee and Essex in the UK, have focused on the ecology of nitrate ammonifiers in estuarine sediments of high nitrate

loadings. The pathway of nitrate ammonification has been regarded as short circuit of the biological nitrogen cycle in nature and waste treatment modules.

2. Nitrate reducing bacterial community in river water and sewage

Rivers, particularly in the mouth, are considered to be water carriage and the sink of pollutants both of point and diffuse sources. Mudflats developed along the river mouth have been characterized as a natural bioreactor, having an extended retention time and periodical exposure to the air owing to the tidal flushing movement. There must be almost the same microorganisms involved in the purification process between wastewater treatment and estuarine sediment systems.

In this study, water samples were collected from a wide variety of habitats in terms of organic pollution, ranging from less polluted river water to raw sewage in Tokyo. Aerobic heterotrophic bacteria were isolated from each sample using nitrate/nitrite broth, and their metabolic patterns, such as nitrate respiration, denitrification and ammonification, were examined.

The results showed that the abundance of nitrate ammonifiers increased as the sites were organically polluted. This suggested that nitrate in the effluent that used to be nitrified with sufficient aeration in sewage works could be returned to ammonia in the receiving waters, thereby the energy for the aeration in the sewage works turned out wasteful. Thus, it would be sensible for decision makers and planners to take the possible contribution of nitrate ammonification into account when evaluating nitrate removal and its effect on the receiving environments.

3. Population dynamics in the activated sludge assemblage

The activated sludge process in the sewage works can be regarded as a continuous culture, being constantly influenced by the inflows. First of all, seasonal fluctuation of the major functional populations was investigated in the Shingashi Treatment Plant in Tokyo for two years. A clear seasonal fluctuation pattern was observed only in the number of nitrifying bacteria, of which growth rate may be highly influenced by environmental temperatures.

An anoxic selector originally designed for phosphorous removal, in the aeration tanks acted as the site for denitrification, since the sewage contained a high concentration of nitrate inputs of an industrial origin. Enrichment of nitrate into the mixed and dialysis cultures revealed that nitrate would induce the specific population changes in the activated sludge microbial community.

4. Nitrate ammonifiers in the nutrified estuarne sediments

According to a classical notion based on previous culture-dependent studies, certain members of fermentative bacteria are said to play an important role in dissimilatory reduction of nitrate to ammonia. But this would not always reflect the whole bacterial community responsible for nitrate ammonification. Molecular-based technique enables us to investigate *in situ* bacterial community structure in the environment independently from cultural procedures.

In this study, four sites in the estuarine sediments of the Colne River in England were chosen to investigate the distribution of the functional gene (*nrfA*) encoding nitrate ammonification by using metagenomic approach such as PCR amplification, TA cloning and DNA sequencing techniques.

Clear evidence of habitat segregation between upper and deeper layers of sediment was obtained by phylogenetic analysis of the environmental clones. Besides, geographical differentiation of two locations, *i.e.* the tidal zone and stable zone, was also observed by the same analysis. This may be resulted from the disturbance caused by tidal actions, forming the turbidity maximum.

A *nrfA* containing isolate (*Aeromonas* sp.) and some *nrfA* clones collected from the Colne estuary clustered with those from the anammox/anaerobic wastewater treatment modules and type cultures such as the *Desulfovibrio* and *Shewanella* groups. The other *nrfA* clones formed distinct clusters; one of which was related to the *Bacteroides* group and the rest formed unknown clusters of supposedly uncultured members of bacteria, which were quite divergent from those in the existing DNA sequence databases.

Although the predominance of *Bacteroides*-related clones was found in the Colne estuary, the relationship with their gene expression and *in situ* activity are still unknown. But the result of habitat segregation may suggest that their distribution pattern should be resulted from their growth and survival under specific conditions in the environment.

5. Nitrate ammonification to be linked with the anammox process

Recently, a hitherto unnoticed reaction, the anaerobic ammonia oxidation (anammox), has been discovered in a waste treatment module. The process depends on the external supplies of ammonia and nitrite as its substrates. This study shall fill a gap which link up with the anammox mystery, which may be occurred in estuarine habitats influenced by human activities (**Figure 5-1**).

Alresford creek, Essex, UK

Figure 5-1, An estuary, as an interface between lands and the sea as well as between nature and the human activities, being expected of purification equivalent to sewage works.

Appendix:

Influence of night soil contamination on activated sludge microbial community in Bangkok, Thailand (Research Note)

Human waste is a major component of sewage rich in nitrogen, but it could be an important source as fertilizer. Since night soil contains a larger amount of organic matter with its small volume, it can remarkably affect biological degradation in conventional wastewater treatment. The objective of this study is to evaluate the influence of night soil on microbial communities in activated sludge fed with two distinct types of domestic wastewater in Bangkok. Most of domestic wastewater in Thailand is typical greywater with less fecal contamination due to the use of traditional Thai-style toilets having leaching cesspools. However, this study showed a large increase in organic loads to greywater, resulting in microfauna and microflora shifts in activated sludge assemblages when receiving human waste as a nitrogen source.

Nitrogen is now recognized as a "public enemy" (Dalton and Brand-Hardy, 2003) because of its high content of excess nutrients that cause severe eutrophication of waters receiving sewage effluent and agricultural runoff. In this study, the effect of nitrogen-rich night soil on microbial community was evaluated by the microflora and microfauna analyses in activated sludge.

Sri Phraya (SP) treatment plant of a contact stabilization process is located in Bangkok, which serves for 120,000 populations. The catchment area of the combined sewers is 2.7 km^2 and the treatment capacity is 30,000m^3/day. Hauy Kwang (HK) treatment plant in Bangkok is specially designed for a local housing estate, whose treatment capacity is 2,400m^3/day and serves for 16,800 populations. The plant has a separate collection system and conventional activated sludge process. Activated sludge from SP treatment plant and influents to both SP and HK treatment plants were used in this experiment. Two distinctive characteristics of water quality as "greywater" for the influent to the SP plant and "black water" (*i.e.* true sewage) for that to the HK plant were confirmed by analyzing BOD, COD, inorganic nitrogen content and fecal coliform counts (data not shown).

One liter of the activated sludge was poured into each of two sets of Imhoff cones. After having settled down the sludge for half an hour, one set was fed with the greywater-dominant SP wastewater and the other one was fed with the night soil-contaminated HK wastewater to fill up to 1 L volume constantly. Both sets were aerated through air bubble-diffusers using an air pump. Activated sludge was maintained using a "fill and draw" technique and incubated at 30^oC. The draining and feeding process on an everyday basis continued for 3 weeks since the microbial communities during acclimation changed dramatically during the first 2 weeks, but remained constant thereafter (Hiraishi *et al.*, 1991). Microflora and microfauna were examined at the initial and the end (21st day) of the experiment.

The activated sludge mixed liquor samples were examined to enumerate

aerobic heterotrophic bacteria (Takii and Konda, 1981), nitrifying and denitrifying bacteria (Nakajima, 1979) and sulphate-reducing bacteria (Mara and Williams, 1971). The spread plate technique was used for heterotrophic counts, whilst the MPN method was adopted for enumerating the other functional populations of bacteria. Thirty colonies of aerobic heterotrophic bacteria were isolated and identified according to the first stage diagnostic table of Cowan and Steel (Barrow and Felthan, 1993). Quantitative examination of the microfauna was carried out using a counting chamber with 1 mm sections, and generic composition of microbial community, consisting of protozoa and other invertebrates, was calculated.

Several bacteria metabolic populations were monitored as shown in **Table A-1**. There was not significant difference observed between two conditions except for nitrifying bacteria and aerobic heterotrophic bacteria. The numbers of both bacteria were one order larger in the sludge fed true sewage than in the control. This may be due to higher organic and nitrogen loads.

Table A-1 Effect of night soil on the bacterial populations in greywater (MPN or CFU/mL)

Metabolic type	0 day	without night soil	with night soil
Ammonia-oxidizing bacteria	$2.4x10^5$	$2.4x10^5$	$1.7x10^6$
Nitrite-oxidizing bacteria	$4.6x10^4$	$1.7x10^5$	$1.3x10^6$
Denitrifying bacteria	$2.4x10^6$	$5.4x10^6$	$3.5x10^6$
Sulphate-reducing bacteria	$2.4x10^4$	$2.4x10^4$	$1.7x10^4$
Heterotrophic bacteria	$8.0x10^6$	$6.0x10^6$	$1.8x10^7$

Shift of aerobic heterotrophic bacterial flora in the two sets is shown in **Table A-2**. A clear difference in the dynamics of Flavobacterium-Cytophaga populations can be observed, namely they predominated in the greywater-fed sludge, but disappeared in the true sewage-fed sludge. The proportion of coryneforms, which is a versatile group of bacteria that utilize a wide range of organic matter, also increased in the same condition. These may be attributed to the influence by night soil contamination. Significant differences of microfauna were also observed between two distinct conditions, as shown in **Table A-3**. Based on this experiment, night soil contamination caused low diversity but predominance of typical activated sludge indicators such as *Vorticella*, a stalked ciliate.

Table A-2 Effect of night soil on the activated sludge microflora

Taxonomic group	0 day	without night soil	with night soil
Bacillus	3	7	10
Coryneforms	3	3	13
Gram positive cocí	7	10	3
Acinetobacter/Moraxella	31	14	37
Alcaligenes	24	23	24
Pseudomonas	3	7	3
Flavobacterium/Cytophaga	13	26	0
Enterobacteriaceae	13	7	0
No growth	3	3	10

(%)

Table A-3 Effect of night soil on the activated sludge microfauna

Genera	0day	without night soil	with night soil
Monas	40	200	80
Peranema	40	0	0
Trachelophyllus	160	0	0
Aspidisca	40	160	0
Trochilia	0	40	0
Litonotus	0	320	0
Coleps	80	0	0
Vorticella	80	40	1120
Vaginicola	0	120	0
Chaetospira	0	40	0
Tokophrya	120	0	40
Euglypha	80	1880	160
Arcella	240	160	120
Centropyxis	80	200	0
Rotaria	40	120	0
Chaetonotus	80	160	0
Aelosoma	0	40	80
Daphnia	40	0	0
Total	1120	3480	1600

(individual/mL)

Microbial communities and their dynamics have been monitored in activated sludge process with special reference to its operational conditions.

The results of this study showed that night soil contamination induced the predominance of *Vorticella* spp., whilst a wide variety of protozoa and metazoan were found in the greywater-based sludge. The results presented here showed that increased nitrifier populations in the night soil-contaminated sludge could be derived from direct influence of nitrogen-rich human excreta. Thus, it is apparent that human waste not only increase organic loads, but also ammonium concentration in the water that could support some populations but inhibit other members, leading to a population shift in response to the loads.

References

Barrow GI and Felthan RKA (1993) *Cowan and Steel's Manual for the Identification of Medical Bacteria*, Cambridge Univ. Press, 331pp.

Dalton H and Brand-Hardy R (2003) Nitrogen: the essential public enemy, *J Applied Ecol*, **40**: 771-781.

Hiraishi A, Morishima Y, Takeuchi J (1991) Numerical analysis of lipoquinone patterns in monitoring bacterial community dynamics in wastewater treatment system, *J Gen Appl Microbiol*, **37**: 57-70.

Mara DD and Williams DJA (1971) The evaluation of media used to enumerate sulphate reducing bacteia, *J Appl Bacteriol*, **33**: 543-552.

Nakajima T (1979) Denitrification by the sessile microbial community of a polluted river, *Hydrobiologia*, **66**: 57-64.

Takii S and Konda T (1981) Influence of dilution rate on the bacterial flora of activated sludge origin in continuous culture systems, *Jpn J Ecol*, **31**: 13-22.