Developing and Optimizing Processes for Biological Nitrogen Removal from Tannery Wastewaters in Ethiopia

Seyoum Leta

Royal Institute of Technology
Department of Biotechnology
Stockholm 2004
Cover photograph
*Brachyomonas denitrificans* (CCUG 45880)
Stained by species-specific 16S rRNA probe

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To my Family
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መግ 115:3

ISBN 91-7283-830-2

Abstract

In Ethiopia industrial effluents containing high contents of organic matter, nitrogen and heavy metals are discharged into inland surface waters with little or no pre-treatment. Significant pollution concerns related to these effluents include dissolved oxygen depletion, toxicity and eutrophication of the receiving waters. This has not only forced the government to formulate regulations and standards for discharge limits but also resulted in an increasing interest and development of methods and systems by which wastewater can be recycled and used sustainably. The need for technologies for environmentally friendly treatment of industrial wastes such as tannery wastewaters is therefore obvious. Biological processes are not only cost effective but also environmentally sound alternatives to the chemical treatment of tannery wastewaters.

The aim of the research presented in this thesis was to develop and optimize processes for biological nitrogen removal from tannery wastewaters and to identify the most efficient denitrifying organisms in tannery wastewaters laden with toxic substances. A pilot plant consisting of a predenitrification anoxic system, aerated nitrification compartment and a sedimentation tank (clarifier) all arranged in series was developed and installed on the premises of Addis Ababa University, Ethiopia. In spite of high influent chromium and sulphide perturbations over the successive feeding phases, the performance of the pilot plant was encouraging. The overall removal efficiency of the pilot plant over the experimental feeding phases ranged from 82–98% for total nitrogen, 95–98% for COD, 96–98% for BOD, 46–95% for ammonia nitrogen, 95–99% for sulphide and 93–99% for trivalent Chromium.

Six isolates from over 1000 pure cultures were identified as the most efficient denitrifying bacteria. From both cellular fatty acid profiles and 16S rRNA gene sequencing, the six selected strains were phylogenetically identified as Brachymonas denitrificans in the β-subdivision of the Proteobacteria. All the six strains contain cd-type nitrite reductase. The efficient isolates characterized in this study are of great value because of their excellent denitrifying properties and high tolerance to the concentrations of toxic compounds prevailing in tannery wastewaters. Bio-augmentation of the pilot plant with this bacterium showed a clear correlation between in situ denitrifying activities measured by nitrate uptake rate, population dynamics of the introduced B. denitrificans monitored by fluorescent in situ hybridization and the pilot plant performance, suggesting that the strategy of introducing this species for enhancing process performance has potential applications.

Moreover, the nitrate-reducing, sulphur-oxidizing bacteria (NR-SOB) were also found in the pilot plant in abundance with steady sulphide removal efficiency during the study period. This could provide opportunities for the application of biologically mediated simultaneous removal of sulphide and nitrogen from tannery effluents. In addition to enriching high consortia of denitrifiers in the anoxic system to attain high denitrification efficiency and also improving the overall nitrification efficiency of the system, the predenitrification-nitrification pilot process plant stimulated the activity of indigenous NR-SOB to simultaneously remove sulphide from the system. Thus, the pilot plant was found to be operationally efficient for the removal of nitrogen, organic matter and other pollutants from tannery wastewaters.

Keywords: Biological nitrogen and sulphide removal, denitrifying bacteria, nitrate-reducing, sulphur-oxidizing bacteria, nitrate uptake rate, fluorescent in situ hybridization, pollution, tannery effluents.
LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which in the text will be referred to by their roman numerals:


IV. **Leta, S., Assefa, F. and Dalhammar, G.** 2004. Enhancing biological nitrogen removal from tannery effluent by using the efficient *Brachymonas denitrificans* in pilot plant operations. Accepted for publication in *World Journal of Microbiology and Biotechnology*.


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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFLP</td>
<td>amplified fragment length polymorphism</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>AUR</td>
<td>ammonium uptake rate</td>
</tr>
<tr>
<td>BOD</td>
<td>biochemical oxygen demand</td>
</tr>
<tr>
<td>BPT</td>
<td>biochemical phenotype</td>
</tr>
<tr>
<td>CCUG</td>
<td>culture collection, University of Gothenburg, Sweden</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>COD</td>
<td>chemical oxygen demand</td>
</tr>
<tr>
<td>DDJB</td>
<td>DNA database of Japan</td>
</tr>
<tr>
<td>DGGE</td>
<td>denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxynucleic acid</td>
</tr>
<tr>
<td>DO</td>
<td>dissolved oxygen</td>
</tr>
<tr>
<td>DS</td>
<td>dissolved solids</td>
</tr>
<tr>
<td>EMBL</td>
<td>European molecular biology laboratory</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Authority/Ethiopian Privitization Agency</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>FAO</td>
<td>United Nations Food and Agriculture Organization</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
</tr>
<tr>
<td>GOE</td>
<td>Government of Ethiopia</td>
</tr>
<tr>
<td>HRT</td>
<td>hydraulic retention time</td>
</tr>
<tr>
<td>MCRT</td>
<td>mean cell retention time</td>
</tr>
<tr>
<td>MLSS</td>
<td>mixed liquor suspended solids</td>
</tr>
<tr>
<td>MLVSS</td>
<td>mixed liquor volatile suspended solids</td>
</tr>
<tr>
<td>MPN</td>
<td>most probable number</td>
</tr>
<tr>
<td>NEQS</td>
<td>national environmental quality standards</td>
</tr>
<tr>
<td>NRB</td>
<td>nitrate-reducing bacteria</td>
</tr>
<tr>
<td>NR-SOB</td>
<td>nitrate-reducing, sulphur-oxidizing bacteria</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>protein database</td>
</tr>
<tr>
<td>Php</td>
<td>phene plate</td>
</tr>
<tr>
<td>RAPD</td>
<td>randomly amplified polymorphic DNA</td>
</tr>
<tr>
<td>RBC</td>
<td>rotating biological contactor</td>
</tr>
<tr>
<td>RDP</td>
<td>ribosomal database project</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SRB</td>
<td>sulphur-reducing bacteria</td>
</tr>
<tr>
<td>SS</td>
<td>suspended solids</td>
</tr>
<tr>
<td>SSU</td>
<td>small subunit</td>
</tr>
<tr>
<td>TDS</td>
<td>total dissolved solids</td>
</tr>
<tr>
<td>TGGE</td>
<td>temperature gradient gel electrophoresis</td>
</tr>
<tr>
<td>TKN</td>
<td>total kjeldahl nitrogen</td>
</tr>
<tr>
<td>UNEP</td>
<td>United Nations Environment Programme</td>
</tr>
<tr>
<td>UNIDO</td>
<td>United Nations Industrial Development Organization</td>
</tr>
<tr>
<td>UPGMA</td>
<td>unweighted-pair group mathematical averages</td>
</tr>
<tr>
<td>WWTP</td>
<td>wastewater treatment plant</td>
</tr>
</tbody>
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5.3.1 The pilot process plant

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5.5.2 The role of nitrate-reducing, sulphur-oxidizing bacteria in the system

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

ACKNOWLEDGEMENTS

REFERENCES

ORIGINAL PAPERS (I-V)
1 INTRODUCTION

With a rapidly expanding population and a growing trend of industry, problems related to the management of wastewaters have become of considerable magnitude in Ethiopia. At present there are more than 20 tanning industries operating in the country and only 10% of the existing tanning industries treat their wastewaters to any degree, while the majority (90%) discharge their wastewaters into nearby water bodies and open land without any form of treatment (EPA, 2003). This now has created concerns at all levels in the country that the rivers could be heavily polluted from the excessive industrial discharges and lead to serious health and ecological consequences. Communities’ downstream areas use the waters from the rivers for a variety of purposes such as drinking, fishing, livestock watering, irrigation, and recreational purposes. It is of necessity that the river waters remain in ‘a healthy natural state’ for both health and ecological reasons.

A wide range of processes and chemicals, including chromium salts, is used in the tanning processes. This gives rise to a wastewater containing high concentrations of nitrogenous compounds, COD (including proteins and fat), BOD5, and high salt content (chromium, sulphides, chlorides, etc.) and which is strongly alkaline (UNEP, 1991, paper I). The predominant N fractions in tannery effluents are organic N, linked to proteins from hides and skins, and ammonia nitrogen. Nitrogen in its various forms can have toxic effects on aquatic life forms, contributes to eutrophication of receiving waters and also becomes a concern if the receiving stream is to be used downstream as a source of drinking water.

This has not only forced the government to formulate regulations and standards for discharge limits (GFE, 2002) but also resulted in an increasing interest in the development of methods and systems by which wastewater can be recycled and used sustainably. The need for technologies for environmentally friendly treatment of industrial wastewaters is therefore obvious. Biological processes are not only cost effective but also environmentally sound alternative to chemical treatment of tannery effluents.

In a wastewater treatment plant, nitrogen can mainly be removed by biological means, and biological nitrogen removal processes are more environmentally friendly than other technologies. Biological nitrogen removal is essentially a two-step process, nitrification followed by denitrification. Nitrification is the biological oxidation of ammonia to nitrite/nitrate by autotrophic nitrifying organisms under aerobic conditions. Denitrification is the dissimilatory reduction of nitrate to molecular nitrogen by heterotrophic organisms using nitrate as an electron acceptor and degradable carbon as energy source under anoxic conditions. In this study, a predenitrification nitrification process was developed and used for biological nitrogen removal from tannery effluents. While the organics in the wastewater provide carbon substrate for denitrification, such a system is also important for the protection of the sensitive nitrifying organisms from shock by toxicants present in tannery effluents, which helps to achieve high degrees of nitrification.

This thesis is presented in six sections including this introduction. Section II describes the environmental impacts of leather industry in Ethiopia. Section III provides an overview of biological nitrogen removal and process variables. Section IV discusses the application of environmental biotechnology to wastewater treatment for increasing process stability and efficiency. Section V outlines the present investigations. Concluding remarks and future perspectives are given in Section VI of the thesis.
2 THE ENVIRONMENTAL IMPACTS OF LEATHER INDUSTRY IN ETHIOPIA

Ethiopia is located between 33° and 48° East longitude, and 3° and 15° North latitude. It has a rugged and mountainous topography with the altitude ranging from a height of 4620 m above sea level at Mount Ras Dejen in North Gondar to a low of 110 m below sea level in the Dalol Depression of the Afar Region. It has an area of 1.130 million km². Sixty-six percent of the total area is estimated to be suitable for agriculture (EPA, 2003). The Great Rift Valley separates the western and northern highlands from the south-eastern and eastern highlands (Figure 1). The rural environment in Ethiopia is endowed with farmlands, lakes, rivers, livestock, forests, woodlands, grasslands, wildlife and plenty of open spaces. Approximately 60 percent of Ethiopia’s land surface is classified as arid and semi-arid, the remaining 40 percent being sub-humid and humid and thus of high agricultural potential.

![Map of Ethiopia showing its topography](image)

**Figure 1.** Map of Ethiopia showing its topography

2.1 Sectoral overview

Like many other developing countries, Ethiopia’s economy is dependent on agriculture. Modern industries became established in Ethiopia towards the end of the 19th century. The leather and leather products sub-sector is increasing in Ethiopia. With abundant livestock resources, Ethiopia has a comparative advantage in producing leather and leather products. According to an FAO (1996) estimate, the country has 30 million cattle, 24 million sheep and 18 million goats, and because of these raw material resources, skins and hides both in the semi-processed and ready to finish levels are one of the major export items of the country. The Ethiopian Privatization Agency (EPA) (2002) estimates that the current contribution of the leather sector to the Ethiopian economy is about 18%, and is the second largest foreign export after coffee. Of the total contribution of the agricultural sector to the economy, livestock accounts for 27% (EPA, 2003).
Even though the country is considered to have huge potential for the growth of leather industry, the economic performance of the sector is limited. The present production is primarily semi-processed hides and skins (at various stages of processing from pickled to crust) for export markets, and only four tanneries are producing finished leather for domestic markets. There are currently more than 20 tanneries in operation with about 17 of the existing tanneries owned by the private sector. Recent data compiled by the EPA (2002) (Table 1) put the current processing capacity of these tanneries at 1.7 million hides, 13 million sheepskins and 18.7 million goatskins per annum.

Table 1. Annual processing capacity of tanneries in Ethiopia

<table>
<thead>
<tr>
<th>No.</th>
<th>Tannery</th>
<th>Hides (Number)</th>
<th>Kg*</th>
<th>Sheep skins (Number)</th>
<th>Kg</th>
<th>Goat skins (Number)</th>
<th>Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Addis Tannery</td>
<td>60,000</td>
<td>1,500,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Awash Tannery</td>
<td>320,000</td>
<td>8,200,000</td>
<td>808,000</td>
<td>2,424,000</td>
<td>2,252,000</td>
<td>4,504,000</td>
</tr>
<tr>
<td>3</td>
<td>Bale Tannery</td>
<td>60,000</td>
<td>1,500,000</td>
<td>600,000</td>
<td>1,800,000</td>
<td>600,000</td>
<td>1,200,000</td>
</tr>
<tr>
<td>4</td>
<td>Blue Nile Tannery</td>
<td>-</td>
<td>-</td>
<td>532,000</td>
<td>1,596,000</td>
<td>1,028,000</td>
<td>2,026,000</td>
</tr>
<tr>
<td>5</td>
<td>Kombolcha Tannery</td>
<td>-</td>
<td>-</td>
<td>1,100,000</td>
<td>3,500,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Dire Tannery</td>
<td>140,000</td>
<td>3,500,000</td>
<td>1,015,000</td>
<td>3,045,000</td>
<td>1,185,000</td>
<td>2,370,000</td>
</tr>
<tr>
<td>7</td>
<td>Dessie Tannery</td>
<td>-</td>
<td>-</td>
<td>1,260,000</td>
<td>3,780,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>ELICO-EPTF</td>
<td>-</td>
<td>-</td>
<td>840,000</td>
<td>2,520,000</td>
<td>1,540,000</td>
<td>3,080,000</td>
</tr>
<tr>
<td>9</td>
<td>Ethiopia Tannery</td>
<td>336,000</td>
<td>8,400,000</td>
<td>1,540,000</td>
<td>4,620,000</td>
<td>2,240,000</td>
<td>4,480,000</td>
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<tr>
<td>10</td>
<td>Hora Tannery</td>
<td>-</td>
<td>-</td>
<td>420,000</td>
<td>1,260,000</td>
<td>840,000</td>
<td>1,680,000</td>
</tr>
<tr>
<td>11</td>
<td>Mersa Tannery</td>
<td>140,000</td>
<td>3,500,000</td>
<td>1,494,000</td>
<td>4,482,000</td>
<td>1,306,000</td>
<td>2,612,000</td>
</tr>
<tr>
<td>12</td>
<td>Modjo Tannery</td>
<td>-</td>
<td>-</td>
<td>844,000</td>
<td>2,532,000</td>
<td>1,656,000</td>
<td>3,312,000</td>
</tr>
<tr>
<td>13</td>
<td>Sho Tannery</td>
<td>-</td>
<td>-</td>
<td>542,000</td>
<td>-</td>
<td>542,000</td>
<td>1,084,000</td>
</tr>
<tr>
<td>14</td>
<td>Walia Tannery</td>
<td>56,000</td>
<td>1,400,000</td>
<td>394,000</td>
<td>1,182,000</td>
<td>476,000</td>
<td>952,000</td>
</tr>
<tr>
<td>15</td>
<td>Six Tanneries under Commissioning</td>
<td>616,000</td>
<td>15,400,000</td>
<td>2,240,000</td>
<td>6,720,000</td>
<td>5,040,000</td>
<td>10,080,000</td>
</tr>
<tr>
<td></td>
<td>Total annual (no.)</td>
<td>1,736,000</td>
<td>-</td>
<td>13,087,000</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total annual (kg)</td>
<td>-</td>
<td>43,400,000</td>
<td>-</td>
<td>39,261,000</td>
<td>-</td>
<td>37,410,000</td>
</tr>
</tbody>
</table>

* For conversions into kg weights, 25kg, 3kg, and 2 kg per number of wet-salted hides, sheepskins and goatskins were used, respectively.

2.2 The environmental challenge

Despite the fact that the number of tanneries is small, their impact in terms of pollution is large. The tanning industry discharges different types of wastes into the environment, primarily in the form of liquid effluents containing organic matter, chromium, sulphide, ammonia and other salts. Ninety percent of the existing leather industries discharge their effluents directly into nearby water bodies and open land without any form of treatment (EPA, 2003).

In leather processing, the conversion of raw hides and skins into leather has three distinct phases: pre-tanning (beam house operations), tanning (using chrome), and finishing steps. A wide range of processes and chemicals, including chrome salts, is used in the tanning and finishing processes (Figure 2). In the pre-tanning step, from which 80% of the BOD load emanates, salt hides are dehaired by soaking them in an aqueous solution of lime, soda and sulphide. This gives rise to a wastewater containing a high COD (including proteins and fat), in addition to sulphate and sulphide. Beamhouse effluents typically have a pH of 11.2 to 12.5 and contain 2.8–4.5 g BOD₅/l and 0.5–1.5 g sulphide/l (UNEP, 1991). In general, beamhouse
wastewaters are mixed with the effluents from other process operation steps. Typical combined tannery wastewater, which has not yet been treated, is characterized by a high BOD<sub>x</sub>, COD, and high salt content (chromium, sulphides, chlorides, etc.) and which is strongly alkaline (UNEP, 1991; paper I, III).

![Diagram of tanning processes and pollutants](image)

**Figure 2.** Schematic of tanning processes and pollutants (adopted from UNEP, 1991)
Based on the total capacity of the 15 operating tanneries in Ethiopia, the estimated volume of wastewater discharges amounts to 2.0–2.5 million m³ per year (UNIDO, 2000) and an estimate of the total load in such a volume of wastewaters are shown in Figure 3.

![Figure 3. Annual pollution loads based on the 15 tanneries production capacities in Ethiopia.](image)

Though environmental pollution has become a fairly serious localized problem in Addis Ababa, water pollution as well as domestic and industrial wastes are some of the problems that have resulted from the process of industrial expansion and social transformation taking place across the country. Pollution becomes more acute when tanneries are concentrated in clusters, as in the case of Akaki and Modjo areas (paper I). Tannery wastewater is highly polluted and the pollutant concentrations observed are many times beyond the limits set by the national environmental quality standards (NEQS) for all wastewater parameters (Leta et al., 2003). Currently, only 10% of the existing tanning industries in Ethiopia treat their wastewaters to any degree, while the majority (90%) discharge their wastewaters into nearby water bodies and open land without any form of treatment (EPA, 2003). Even those with effluent treatment plants have only primary treatment process. With the exception of the Ethiopia tannery, all do not have biological waste treatment processes. A key role is played by the absence of policies to regulate the establishment and management of tanning industrial plants. This has resulted in the misconception that industry is not the motor of development for the area where it is located, but that it produces environmental problems and adversely affects local people living nearby tanneries.

Presently, well-established standards for the discharge of tannery and other effluents are being put in place (EPA, 2003) and new tanning projects cannot be permitted without an effective effluent treatment plant. The national legislation on environmental pollution proclamation (GOE, 2002) urges industries to meet the minimum permissible limits to cope with sustainable industrial development in the country.
3 BIOLOGICAL NITROGEN REMOVAL AND PROCESS VARIABLES

3.1 Nitrogen: Environmental and wastewater concerns

In wastewaters, nitrogen may be found in four forms:

- Organic nitrogen (urea, amines, proteins, faecal materials)
- Ammonia nitrogen (NH₃-N or NH₄-N)
- Nitrate nitrogen (NO₃-N) and
- Nitrite nitrogen (NO₂-N)

The presence of nitrogenous or nitrogen-containing wastes in tannery effluent can adversely impact or pollute the quality of the receiving water. Significant pollution concerns related to the presence of nitrogenous wastes include dissolved oxygen depletion, toxicity, eutrophication, and methemoglobinemia (Table 2).

**Table 2. Environmental concerns of nitrogen pollution**

<table>
<thead>
<tr>
<th>Nitrogenous compounds</th>
<th>Pollution concerns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia nitrogen</td>
<td>overabundant growth of aquatic plants, dissolved oxygen depletion, toxicity as NH₃</td>
</tr>
<tr>
<td>Nitrite nitrogen</td>
<td>overabundant growth of aquatic plants, dissolved oxygen depletion, toxicity</td>
</tr>
<tr>
<td>Nitrate nitrogen</td>
<td>overabundant growth of aquatic plants, dissolved oxygen depletion, toxicity and Methemoglobinemia</td>
</tr>
</tbody>
</table>

Ammonia nitrogen discharge into a receiving stream will undergo the process of oxidation in the presence of dissolved oxygen and nitrifying bacteria as shown in Figure 4. This will cause the depletion of DO in receiving water that is available to sustain the aquatic life. Second, since the different forms of nitrogen (NH₄⁺, NO₂⁻ and NO₃⁻) serve as nitrogen nutrients for the growths of aquatic plants, especially algae, DO is depleted from the receiving water by microbial activity to decompose the dead plants. Moreover, all the three forms of nitrogen cause toxicity to aquatic life. Although ammonium ions are the preferred nitrogen nutrient for most organisms, ammonium ions are converted to free ammonia with increasing pH. Free ammonia is toxic to aquatic life.

The discharge of nitrate nitrogen in excess promotes eutrophication in the receiving waters. Nitrate in drinking water above the maximum containment level (MCL) of 10 mg/l can also cause a human health condition called methemoglobinemia (Cheremisinoff, 1996; Gerardi, 2002). This disease is usually known as “blue baby syndrome”, which refers to the disease experienced by infants, which consume ground water contaminated with nitrate nitrogen. Upon consuming nitrate-contaminated groundwater, the nitrate ion is quickly reduced to nitrite in the infant’s digestive tract and then binds quickly and tightly to the haem iron within the red blood cells. This prevents the haemoglobin from binding and transporting oxygen throughout the infant’s body and causes the infant’s skin to turn blue, hence the term “blue
baby syndrome.” Methemoglobinemia usually is associated with rural communities where potable water is obtained from groundwater.

To reduce the adverse impacts of nitrogenous wastes upon the receiving water, biological nitrogen removal by nitrification and denitrification processes is required to lower the level of nitrogenous wastes in its final effluent. A nitrification requirement usually is issued as ammonia (NH₃-N) discharge limit; a denitrification requirement usually is issued as total nitrogen or total kjeldahl nitrogen (TKN) discharge limit (Table 3). Total nitrogen is the combination of ammonia, nitrate/nitrite and organic nitrogen.

Table 3. Permit requirements for nitrification and denitrification

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Description</th>
<th>nitrification/denitrification</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₃-N</td>
<td>Ammonia N</td>
<td>nitrification</td>
</tr>
<tr>
<td>NH₄-N</td>
<td>Ammonium N</td>
<td>nitrification</td>
</tr>
<tr>
<td>NBOD</td>
<td>Nitrogenous biochemical</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxygen demand</td>
<td>nitrification/denitrification</td>
</tr>
<tr>
<td>TKN</td>
<td>Total Kjeldahl nitrogen</td>
<td>nitrification/denitrification</td>
</tr>
</tbody>
</table>

Because of increased environmental concerns, biological nitrification and denitrification requirements will play a major role in the treatment of nitrogenous wastes in activated sludge processes treating tannery effluents in Ethiopia.

3.2 Biological nitrogen removal technologies/processes

Over the last 10 decades, many different technologies for wastewater treatment have been developed, all of which include biological treatment technologies at some stage, with the task of mitigating the destructive effect of man’s wastes on nature. Increased eutrophication problems in major seawater bodies in Europe in the 1980s led to the development of improved technologies for biological wastewater treatment. Subsequently, increased demands on wastewater treatment efficiencies required a better understanding of the biological processes that were the basis of its treatment. Since then, much has been developed scientifically and the development is still in progress (Stams et al., 1997). In the field of wastewater treatment, it is generally accepted that biological treatment is less energy-intensive, cheaper and environmentally friendly. For biological treatment, wastewater is a medium for organisms, which work best under constant and steady conditions. An optimum environment with nutrients of known composition are rarely available in a given wastewater environment. The main problem with tannery wastewater treatment therefore arises from the variability of its composition with respect to amount, nutrients, toxicants and or inhibitors.

Depending on the characteristics of the waste streams and the effluent treatment objectives, various process configurations for biological nitrogen removal have evolved over the past twenty years (Cioe and Muyima, 1997; Metcalf & Eddy, 2003). The main processes for biological nitrogen removal are based on how the microbial biomass is maintained in the system, as a fixed film or in a suspension, usually as an activated sludge. In a fixed biofilm process, the microorganisms are attached to a support and are exposed to the wastewater to be treated (Boller et al., 1994). Trickling filters submerged or expanded bed aerobic filters and rotating biological contactors have also been used for carbon removal and/or nitrification (Barnes and Bliss, 1983; Chereuisinoff, 1996; Metcalf & eddy, 2003). In trickling filter, the microorganisms are immobilized on a support medium such as rocks, wood slats or plastic
sheets, over which wastewater are trickled. In rotating biological contactors (RBC), packed circular disks submerged in wastewater are rotated slowly to allow bacterial growth on the surface of the disk to form a slime layer. The disks contact wastewater and air as they rotate. In submerged up-flow packed bed reactors, stones, gravel or plastic materials are used where microbial growth can also be attached to media in a packed bed. Wastewater is made to flow to the reactor and air or oxygen is introduced into the wastewater.

The activated sludge process is the most commonly applied biological process for the treatment of both municipal and industrial wastewaters. The majority of suspended growth biological nitrogen removal systems in operation today are activated sludge processes with aerobic zones for carbon removal and nitrification, and anoxic zones for denitrification. In processes such as the modified Ludzack Ettinger Process (predenitrification zone upstream of the nitrification zone), nitrified mixed liquor is recycled from the aerobic zone to the anoxic zone, where it is denitrified using the incoming wastewater as a carbon source. High degrees of total nitrogen removal have been reported using these systems from both municipal (Im et al., 2001) and industrial wastewaters (Hirata et al., 2001). In processes with a post-denitrification zone, the denitrification rates are significantly lower.

The pre-denitrification-nitrification process is particularly suitable for the treatment of industrial wastewaters laden with toxic substances such as those from tanneries containing high concentrations of degradable organic carbon, nitrogen and ammonia. This process allows the optimal use of the incoming wastewater as carbon source for denitrification, protects the sensitive nitrifying organisms from toxic shocks, and helps to achieve high degrees of nitrification. The process relies on a dense microbial population mixed in suspension with the wastewater under aerobic conditions.

3.3 Microbial transformation of nitrogen

Microorganisms play a major role in nitrogen cycling in the environment. The nitrogen cycle consists of five processes: ammonification, nitrification, denitrification, nitrogen fixation and assimilation (Gerardi, 2002). Depending on the kind of nitrogen compounds present in wastewater, nitrogen removal requires up to three processes in sequence: ammonification, nitrification and denitrification (Figure 4).

Nitrogen containing bio-molecules (R-NH₂) are first converted to ammonia by bacterial decomposition and hydrolysis through a process called ammonification. This process is driven by a wide variety of microorganisms in wastewaters. The biological oxidation of ammonium (NH₄⁺) to nitrite (NO₂⁻) and then nitrate (NO₃⁻) is sequentially accomplished by nitrifying bacteria. Ammonium and nitrite exist in wastewaters but are unstable molecules that readily accept oxygen, generating nitrate as a dominant form of nitrogen in aerated wastewaters (Gerardi, 2002). Nitrate can undergo assimilatory and dissimilatory reductions as it is converted to ammonia and molecular nitrogen, respectively.

Denitrification is a dissimilatory nitrate reduction whereby certain species of facultative and anaerobic organisms reduce nitrate and nitrite to molecular nitrogen or nitrogen oxides (Zumft, 1992; Bitton, 1999; Gerardi, 2002). The production of nitrogen gas connects denitrification to the nitrogen cycle via nitrogen fixation. Denitrification constitutes one of the main parts of the global nitrogen cycle undertaken by bacteria. More recent concerns related to denitrification begin to foster research in this area. Nitrate, irrespective of its role as essential nutrient, has become a pollutant of ground and surface waters, causing a major problem for the supply of drinking water. N₂O is next to CO₂ and CH₄ in its importance as a potent greenhouse gas and, together with NO, is of much concern in terms of the ozone chemistry of the atmosphere (Ye et al., 1994).
Activated sludge processes treating industrial wastes such as tannery effluents receive large quantities of organic-nitrogen wastes that release ammonia in the wastewater stream as it is hydrolysed and deaminated by heterotrophic bacterial activity. Ammonia in the form of ammonium ion within the activated sludge process has several fates. It can be used as nutrient source for nitrogen by organotrophs and nitrifying bacteria. It can also be air-stripped to the atmosphere as ammonia at high pH, and under appropriate conditions, it is oxidized to nitrite by ammonia oxidizing bacteria. If none of these happen, effluent ammonia will be high. Although ammonium is removed as nutrient source for nitrogen, its concentration increases as organic nitrogen wastes are deaminated. As the quantity of organic nitrogen waste decreases, the quantity of ammonium ion increases.

In the process of microbial nitrogen transformation in wastewater treatment, nitrogen is found in seven oxidation states (Table 4). Nitrogen always enters biosynthetic pathways (processes for producing cellular material) in an inorganic form and oxidation state of $-3\ (\text{NH}_2^-)$. Ammonium is the preferred nutrient source of nitrogen. When ammonium is in less supply, nitrate can be used by bacteria through a process referred to as assimilatory nitrate reduction (Figure 4). When nitrate is used as the nitrogen nutrient, the nitrate is first reduced.
to ammonium in which it is assimilated into new cellular material such as amino acid and proteins (organic nitrogen).

\[
\text{NO}_3^- \rightarrow \text{NH}_4^+ \rightarrow \text{organic nitrogen} \quad \text{(Assimilatory nitrate reduction)} \tag{1}
\]

Nitrate in wastewater is a necessary constituent for the production of new cellular material. Approximately 14% of the nitrogen entering the activated sludge process is used in cellular growth and reproduction. Nitrogen is the major constituent of proteins and nucleic acids (genetic material) of all cells, and nitrogen is found in peptidoglycans, the rigid cell wall layer of most bacteria. An additional 10% to 15% of nitrogen entering the activated sludge process is removed in the wasting of excess sludge. The remaining nitrogen is discharged to the receiving water, nitrified, denitrified, or air stripped to the atmosphere (Bitton, 1999; Gerardi, 2002).

**Table 4. Nitrogenous compounds produced in biological wastewater treatment processes**

<table>
<thead>
<tr>
<th>Nitrogenous compound</th>
<th>Chemical formula</th>
<th>Oxidation states of N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>NO$_3^-$</td>
<td>+5</td>
</tr>
<tr>
<td>Nitrite</td>
<td>NO$_2^-$</td>
<td>+3</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>NO</td>
<td>+2</td>
</tr>
<tr>
<td>Nitrous oxide</td>
<td>N$_2$O</td>
<td>+1</td>
</tr>
<tr>
<td>Molecular nitrogen</td>
<td>N$_2$</td>
<td>0</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>NH$_2$OH</td>
<td>-1</td>
</tr>
<tr>
<td>Ammonia/ammonium</td>
<td>NH$_3$/NH$_4^+$</td>
<td>-3</td>
</tr>
</tbody>
</table>

3.4 **Nitrification Process**

Since the nitrogen in the influent of a WWTP is present either in a form of urea, amino acids, and proteins which undergo hydrolysis and deamination that release ammonium ions, the nitrifying bacteria play a central role in nitrogen removal in WWTPs. It is important to lower the ammonia concentrations in the effluent of WWTPs since this compound is toxic to aquatic life and promotes eutrophication in the receiving water bodies. The first step in the biological nitrogen removal processes is nitrification - the aerobic oxidation of ammonium to nitrite (eq.2) by ammonia oxidizing bacteria and the subsequent oxidation of the produced nitrite to nitrate (eq.3) by nitrite oxidizing bacteria.

\[
\text{NH}_4^+ + 1.5 \text{O}_2 \xrightarrow{\text{Ammonia oxidizing bacteria}} \text{NO}_2^- + 2\text{H}^+ + \text{H}_2\text{O} \tag{2}
\]

\[
\text{NO}_2^- + 0.5 \text{O}_2 \xrightarrow{\text{Nitrite oxidizing bacteria}} \text{NO}_3^- \tag{3}
\]

The oxidation of NH$_4^+$ to NO$_2^-$ and then NO$_3^-$ is exergonic, and the nitrifying organisms use this energy to assimilate CO$_2$. Although ammonium and ammonia are reduced forms of nitrogen, that is, are not bonded to oxygen, it is the ammonium, not ammonia, that is oxidized during nitrification. The quantities of ammonium and ammonia in aeration tank are dependent on the pH and temperature of the activated sludge. In the temperature range of 10°C to 20°C.
and pH range of 7.0 to 8.5, which is typical of most activated sludge processes; about 95% of the reduced form of nitrogen is present as ammonium (Gerardi, 2002; paper III, IV).

3.5 The nitrifying bacteria

Nitrifying bacteria belong to the family *Nitrobacteriaceae*. There are several genera of nitrifying bacteria. The genera can be grouped according to those that oxidize ammonia and those that oxidize nitrite (Table 5). Ammonia oxidizers belong to the genera *Nitrosomonas, Nitrosococcus, Nitrosolobus, Nitrospira*, or *Nitrosovibrio*. The second step of nitrification, the oxidation of nitrite to nitrate, is performed by nitrite oxidizing bacteria belonging to the genera *Nitrobacter, Nitrococcus, Nitrospira and Nitrospina* (Prosser 1989; Bitton, 1999; Gerardi, 2002; Madgian et al., 2002). Nitrifiers are generally lithothrophic organisms using carbon dioxide as the main carbon source for growth. Molecular-based techniques showed that a high diversity of ammonium oxidizing bacteria belong to the β- and T- subdivisions of the *Proteobacteria* (Wagner et al., 1995; Juretschko et al., 2002).

**Table 5. Genera of nitrifying bacteria**

<table>
<thead>
<tr>
<th>Energy substrate</th>
<th>oxidized product</th>
<th>genera of nitrifying bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4^+$</td>
<td>NO$_2^-$</td>
<td><em>Nitrosococcus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nitrosocystis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nitrosomonas</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nitrospira</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nitrosolobus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nitrosovibrio</em></td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>NO$_3^-$</td>
<td><em>Nitrobacter</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nitrococcus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nitrospira</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nitrospina</em></td>
</tr>
</tbody>
</table>

It is estimated that approximately 3% to 10% of the bacteria in the activated sludge process are nitrifiers, while the remaining 90% to 97% of the bacteria consist of organotrophs (Gerardi, 2002). The difference in population size between nitrifying bacteria and organotrophs in the activated sludge process is due to two reasons. First, in most municipal and industrial activated sludge processes, the concentrations of carbonaceous wastes (cBOD) greatly exceed the concentrations of nitrogenous wastes (nBOD). Therefore, more substrate is available for the growth of more organotrophs. Second, heterotrophs obtain more energy for reproduction than do nitrifying bacteria when they oxidize their respective substrates (Bitton, 1999; Gerardi, 2002).

Due to the slow growth of nitrifiers in the activated sludge process, nitrifying bacteria are able to increase in number only if their reproductive rate is greater than their removal rate through sludge wasting and discharge in the final effluent (Cheremisinoff, 1996; Bitton, 1999; Gerardi, 2002). Therefore, a high mean cell retention time (MCRT) is required to increase the number of nitrifying bacteria in the activated sludge process (Sandén et al. 1996; Cloete & Muyima, 1997; Paper III). Nitrifying bacteria can be cultivated on selective culture media. However, due to the low generation time of nitrifying bacteria, their poor isolation and slow
Sevoum Lee

colony development on solid media, it is often difficult to identify nitrifying bacteria on selective laboratory media.

In WWTPs, FISH results indicated that some WWTPs are dominated by a single ammonia oxidizers (Juretscko, et al., 1998) while others harbour at least five different co-existing ammonia oxidizing populations that are present in significant numbers (Daims et al., 2001). Traditionally, Nitrospira was considered as the most important nitrite-oxidizer in WWTP (Sandén et al. 1996; Henze et al., 1997). However, using FISH with specific 16S rRNA-targeted probes, Nitrospira-like nitrite-oxidizing bacteria were detected in nitrifying WWTPs (Juretschko et al., 1998; Okabe et al., 1999; Daims et al., 2000, 2001; Gieske et al., 2001). It has been postulated that the predominance of Nitrospira-like bacteria over Nitrobacter in most WWTPs is a reflection of their different survival strategies. While Nitrospira-like nitrite oxidizers are K-strategists and thus may possess a low µmax but are well adapted to low nitrite and oxygen concentrations. Nitrobacter is postulated to be a relatively fast growing r-strategist with low affinities to nitrite and oxygen concentrations (Schramm et al., 1999). Since nitrite concentrations in most reactors from WWTPs are low, Nitrospira will out-compete Nitrobacter in these systems.

3.6 Environmental factors influencing nitrification

Several environmental factors such as dissolved oxygen, pH, temperature, organic concentration, and the presence of inhibitory substances govern nitrification in wastewater treatment processes (Prosser, 1989; Cheremisinoff, 1996; Grunditz et al. 1998; Bitton, 1999; Jonson et al., 2000; Gerardi, 2002). Autotrophic nitrifying organisms are obligate aerobes. Dissolved oxygen concentrations above 1.5 mg/l are essential for nitrification to occur (Gerardi, 2002). Studies show that in order to ensure that dissolved oxygen (DO) is not a limiting factor in the nitrification process, a level not less than 2 mg/l need to be maintained (Cheremisinoff, 1996; Bitton; 1999; Cao et al., 2002; paper III). DO levels below this will significantly reduce the nitrification rate (paper III).

Nitrification, like most bacterial processes, is affected by pH conditions. Nitrification in activated sludge processes begins to accelerate above pH 6.7 and narrow optimal range between pH 7.2 to 8.6 exists (Cheremisinoff 1996; Bitton, 1999; Grunditz et al., 1998; Im et al., 2001; Gerardi, 2002). Systems acclimatized to maintain pH conditions at desired levels have shown to successfully been nitrified (paper III). Low pH in wastewater has a primary effect on nitrifying bacteria by inhibiting enzymatic activity and a secondary effect on the availability of alkalinity.

Since nitrification reactions tend to decrease pH that would likely affect the nitrification rate, the alkalinity of the wastewater is an important consideration. During nitrification of ammonium the alkalinity of the wastewater increase slightly, due to CO2 consumption for autotrophic growth (pH increase), while on the other hand nitrite acid formation from ammonia will cause a decline in pH. If the buffer capacity of a wastewater is weak, the pH will drop far below 6.7 and thus prevent further nitrification by autotrophic nitrification (Gerardi, 2002). Nitrification occurs over a wide range of temperatures, from 8-30°C. The optimum temperature for the growth rate of nitrifiers has been reported to be around 30°C (Bitton, 1999; Grunditz et al., 1998; Gerardi, 2002).

The organic loading rate to the aeration tank also affects the efficiency of nitrification as this favours the growth of organotrophs/heterotrophs. Due to their slow growth autotrophic nitrifiers cannot successfully compete with heterotrophic bacteria for oxygen. In a highly loaded activated sludge system, the autotrophic nitrifiers will be overgrown by the heterotrophic sludge flora, which consumes the oxygen. High organic loads accelerate the
heterotrophic growth rate and the quantities of sludge produced in the system resulting in a
decrease of the fraction of nitrifiers present in an activated sludge system (Cheremisinoff,
1996). Shock organic loads (increases in influent COD loading) to nitrifying sludge have been
observed to inhibit nitrification (paper III).

Nitrifying bacteria are sensitive organisms, which are extremely susceptible to a wide
variety of inhibitors, found in wastewater. The presence of excess organic matter in
wastewater may be inhibitory due to O₂ depletion by heterotrophs. Various inorganic agents
such as cyanide, thiourea, phenol, anilines and heavy metals can affect the growth and activity
of these organisms. High concentrations of ammonia and nitrous acid can also be inhibitory
(Grunditz et al., 1998; Bitton; 1999; Jansen and Jönsson, 2001; Gerardi, 2002).

3.7 Denitrification

Denitrification is the reduction of oxidized nitrogen compounds like nitrite or nitrate to
gaseous nitrogen compounds by various chemoorganotrophic, lithoautotrophic, and
phototrophic bacteria under anoxic conditions (Figure 4). This metabolic process is also
termed nitrate dissimilation or dissimilatory denitrification. Dinitrogen is the main end
product of denitrification while the nitrogenous gases, nitric and nitrous oxides occur as
intermediates in low concentrations (Tiedje, 1988; Zumft, 1992; Ye et al., 1994; Zumft,
1997). However, these gases are also released as end products when denitrification enzymes
are not completely expressed, e.g., when the concentration of dissolved oxygen is too high
(Tiedje, 1988; Körner and Zumft, 1989). Reduction of nitrate is carried out according to the
following sequence employing different enzymes:

\[
\text{NO}_3^- \xrightarrow{\text{nitrates reductase}} \text{NO}_2^- \xrightarrow{\text{nitrates reductase}} \text{NO} \xrightarrow{\text{nitrates reductase}} \text{N}_2\text{O} \xrightarrow{\text{nitrates reductase}} \text{N}_2
\]

(4)

Complete denitrification is a multi-step process, requiring four separate enzymes for the
reduction of nitrate to nitrogen gas (eq.4). The first step of denitrification, the two-electron
reduction of nitrate to nitrite, is catalysed by nitrate reductase; a molybdenum containing
membrane protein whose synthesis is repressed by molecular oxygen (Zumft, 1997; Madgian
et al., 2002). Nitrite reduction is the defining reaction of denitrification, the step that
differentiates denitrification from other forms of nitrate metabolism. There are two types of
dissimilatory nitrite reductase that catalyse the conversion of nitrite to nitric oxide in bacteria.
One type is the cytochrome cd, nitrite reductase, and the other type is the copper-Containing
nitrite reductase (Ye and Thomas, 2001, Zumft, 1997).

3.8 Denitrifying bacteria

The ability to use nitrate nitrogen, as the final electron acceptor in biochemical reactions is
wide spread among the activated sludge microorganisms. At least 40 species of aquatic
microorganisms can denitrify (Cloete & Muyima 1997). Denitrification is a facultative trait
possessed by a diversity of bacteria belonging taxonomically to the various subclasses of the
Proteobacteria. Denitrification also extends beyond the bacteria to the Archaea, where it is
found among the halophilic and hyperthermophilic branches of this kingdom and may have
evolutionary significance (Zumft, 1992; 1997; Madgian et al., 2002). Most denitrifiers are
heterotrophs/organotrophs and thus need organic compounds as carbon and energy sources.
Some denitrifying bacteria, for example, Microvulva aerodenitrificans (Patureau et al.,
1998) is known to be an aerobic denitrifying organism. In addition, many denitrifying bacteria
can grow by fermentation. Thus, denitrifying bacteria are quite metabolically diverse in terms of alternative energy-generating mechanisms. The common denitrifiers in wastewater treatment plants are chemoorganotrophic bacteria belonging to several genera such as *Pseudomonas, Paracoccus, Alcaligenes, Micrococcus, Achromobacter, Bacillus, Brevibacterium, Flavobacterium*, and *Moraxella* (Cloete & Muyima, 1997; Magnusson et al. 1998; Gerardi, 2002; Metcalf & Eddy, 2003).

With the use of modern molecular tools, identification of more denitrifying strains capable of denitrification seems likely. Recently, *Brachymonas denitrificans* (Leta et al., 2004, Paper II) and *Comamonas denitrificans* (Gumaelius et al., 2003) were isolated and identified as the efficient denitrifiers in activated sludge systems.

### 3.9 Environmental factors affecting Denitrification

For denitrification to occur, the following environmental conditions are necessary:

- **Nitrate/nitrite nitrogen concentrations**: Since nitrate/nitrite serves as an electron acceptor in denitrification, the maximum growth rate of denitrifying bacteria is dependent on the availability of nitrate/nitrite.
- **Anoxic conditions**: The presence of oxygen prevents the formation of the enzyme necessary for the substitution of nitrate for oxygen as the terminal electron acceptor.
- **Presence of organic matter (energy source)**: The presence of organic carbon or suitable electron donor is important prerequisite for denitrification to proceed. The carbonaceous energy source for denitrification can either be internal (organic material naturally present in wastewater), external (e.g. methanol added to the denitrification stage of the process), or self generated (nutrients released through the death of organisms in the process).
- **pH value**: In wastewater, denitrification is most effective at pH between 7.0 and 8.5 (Bitton, 1999; Grunditz et al. 1998; Gerardi, 2002; Metcalf & Eddy, 2003). Alkalinity increases following denitrification. The predenitrification condition maintained the pH during the nitrification process in the present study (Paper III, IV, V).
- **Temperature**: Denitrification may occur over a wide range of temperature from 5°C to 50°C, but is slowed at lower temperatures (Cheremisinoff, 1996; Grunditz et al. 1998; Bitton, 1999; Gerardi, 2002).
- **Inhibitory substances**: Denitrifying organisms are less sensitive to toxic chemicals than are nitrifiers (Gumaelius et al. 1996; Grunditz et al., 1998; paper II, III).

The diversity of denitrifying and nitrifying microbial species in an activated sludge system generally is the result of the composition of the influent wastewater, environmental parameters such as pH and temperature, the process conditions prevailing in the reactor or plant such as mean solid retention time (sludge age), hydraulic retention time, dissolved oxygen and substrate (pollutant) concentrations.
4 ENVIRONMENTAL BIOTECHNOLOGY FOR WASTEWATER TREATMENT PROCESS

4.1 An overview

Biotechnological processes to protect the environment have been used for almost a century now, even longer than the term “biotechnology”. Municipal sewage treatment plants and filters to purify town gas were developed around the turn of the century. Even though little was known originally about the biological principles underlying their function, our knowledge base has increased enormously since that time. As recently as the last decade, the use of molecular tools has allowed the accurate determination of the composition and dynamics of activated sludge and biofilm microbial communities.

Microorganisms are the biological systems, which are generally used in biotechnology for the reduction of pollution from aquatic or terrestrial systems. Bioremediation of contaminated sites and waste treatment in activated sludge bioreactors relies on microbial communities to transform and remediate undesirable industrial wastes. Bioremediation techniques can be used to reduce or remove hazardous waste, which has already polluted the environment. They can also be used to treat waste streams before they leave production facilities, in specialized end-of-pipe-processes. Increasing industrial pollution has led to a greater need for processes that remove specific pollutants such as nitrogen and phosphorus, heavy metals and chlorinated compounds. Biological methods include aerobic and anaerobic processes in fixed bed filters and in bioreactors in which the materials and microbes are held in suspension, the activated sludge process (Metcalf & Eddy, 2003).

Novel biological solutions to environmental problems are fast becoming available through the application of modern biotechnology and in-depth studies of specific natural systems. The predominant reason why so many environmental technologies prefer to rely on microorganisms is that microbial bioconversion is relatively inexpensive, flexible and microorganisms can adapt to variable conditions (self regulation) and also to new molecules. Moreover, environmental biotechnology is perceived as ‘sustainable.’ It may then be argued that the wider scope of biotechnology, as a research discipline and market oriented science-based endeavour, is that of the recently emerging field of environmental biotechnology.

Not all of the microorganisms in an ecological system will be crucial for achieving the desired biochemical transformations. Therefore, it is important to understand the dynamics of this active community and to develop ways of detecting its presence and measuring its activity. Molecular methods developed on the basis of 16S rRNA gene sequences and relevant structural genes for detecting and quantifying specific phylogenetic groups provide ways of monitoring the microbial populations of environmental systems (Amann, 1995).

Significant progress in environmental microbiology includes the development and implementation of molecular tools for determination of the structure and function of microbial communities (Amann et al., 1991). The second comprises the development of methodologies that allow the study of multicellular biofilm consortia (Wagner and Loy, 2002). It is now possible by means of molecular methods such as denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH) to rapidly screen for microbial species (Amman et al., 1995; Jurrestschko et al., 2002).

An understanding of the structure and function of microbial communities in activated sludge and biofilm reactors can be used to enhance the efficacy and stability of biological wastewater treatment systems. Moreover, the identification of links between microbial community composition and function allows for process stability and improved bioaugmentation strategies. The development of combined in situ identification and functional
analysis of individual cells and species allows the specific assignment of nitrogen removal to previously unknown bacteria (paper II, IV).

4.2 Molecular methods for microbial community studies in wastewater treatment processes

Traditionally, identification of microorganisms required the isolation of pure cultures and the identification of physiological and biochemical traits. These microbial consortia have mostly been analyzed by culture-dependent methods such as viable plate count or most probable number (MPN) techniques (Wagner and Amann, 1997; Madigan et al., 2002). These techniques, apart from being laborious and slow, have the drawback of “enrichment bias” and lack sufficient sensitivity and selectivity for poorly or slowly growing microorganisms (Amann et al., 1995; Madigan et al., 2002). Moreover, questions like micro-scale distribution and in situ activity of microorganisms are difficult to address by classical methods (Wagner et al., 1993). Often the most important microbial populations are difficult to cultivate. Culture-dependent methods underestimate the true picture of the composition of microorganisms in complex microbial communities (Amann et al., 1995).

During the last decade, advances in molecular biology and phylogeny analysis techniques have provided new insights into microbial ecology (Raskin et al., 1994; Amann, et al., 1995; Sahn et al., 1999; Schramm and Amann, 1999; Jurestscsco et al., 2002). Molecular approaches developed to study microbial communities are based on the detection and analysis of the small subunit ribosomal RNA (rRNA) molecules and genes (16S rRNA molecules for prokaryotes and 18S rRNA for eukaryotes) (Amann et al., 1998). Today a combination of direct retrieval of rRNA sequences by the polymerase chain reaction (PCR) and fluorescent probing in situ enables the identification and phylogenetic characterization of microorganisms without cultivation (Amman et al., 1991; Spring et al., 1992). For the monitoring of microbial community dynamics, these technical tools can be grouped into two categories: molecular probes, which are used more efficiently when there is previous knowledge of the targeted microbial populations, and DNA fragment analysis, which does not necessitate prior knowledge of microbial community structure before its study (Figure 5).

4.2.1 DNA fingerprinting

A variety of DNA fingerprinting techniques can be used to rapidly differentiate closely related environmental strains. DNA fingerprinting techniques typically are used not to specifically identify environmental isolates, but rather to demonstrate small genetic differences or similarities for population genetics studies, or for diversity studies within closely related species or strains.

The oldest of these approaches, restriction fragment length polymorphism (RFLP) analysis (Owen et al., 1985; Vilgalys and Hester, 1990), uses restriction endonucleases to digest purified DNA from individual strains in order to identify polymorphisms within individual genes. Polymorphisms, or differences within a specific gene, may result in a different number of sites that are recognized by the restriction endonucleases used in each digestion. Such differences can be caused by single base pair changes or by large changes such as insertions, deletions, and rearrangements. Fragments within the digested DNA are separated by agarose gel electrophoresis, transferred to a DNA binding membrane, and detected by hybridization with a labelled probe specific for the gene(s) of interest. Differences or similarities in the numbers and sizes of fragments from each isolate can then be identified.
Figure 5. Approaches towards analysis of microbial community structure with molecular probes and community fingerprinting (adopted from Wagner and Amann, 1997; Stams et al., 1997; Dabert et al., 2002).

Polymerase chain reaction (PCR) (Saiki et al., 1988) technology is widely used in microbial biotechnology. In random amplified polymorphic DNA (RAPD) analysis (William et al., 1990; Hadrys et al., 1992; Sambrook et al., 1989), arbitrary primers (AP-PCR) produce a pattern that can be used to differentiate between closely related bacterial isolates. Unlike RFLP analysis, which is designed to identify differences within specific genes, AP-PCR techniques screen for differences between entire genomes. AP-PCR is much faster than RFLP analysis because it is PCR based and no digestion with restriction endonucleases or hybridization is required. PCR amplified rDNA can also be analysed by amplified fragment length polymorphism (AFLP) (Janssen et al., 1996) to determine group- and species-specific patterns.

In recent years the use of fingerprinting methods has become popular in molecular microbial ecology as an alternative to the more laborious analysis of 16S rRNA gene libraries (Muyzer and Smalla, 1998). Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are some of DNA/RNA fingerprinting methods used to investigate microbial communities (Muyzer et al., 1993; Muyzer and Rensing, 1995; Muyzer and Smalla, 1998; Delbes et al., 2000). With these electrophoresis methods, DNA fragments of the same length, but with different nucleotide sequences can be separated. The separation is
based on the difference in melting temperatures between the amplified DNA sequences and the slower mobility of partially melted amplified fragments compared with intact fragments. DGGE has been applied to study microbial community complexity (Felske et al., 1996; Muyzer et al., 1993). With this technique, the diversity of a microbial population in wastewater treatment systems can be analyzed by comparing fingerprinting patterns. It also allows elution of specific bands of interest from gels followed by sequencing to determine the corresponding bacterial species. By designing a FISH probe based on eluted and cloned sequence, the actual abundance of a gel band that appears to be dominant after DGGE analysis can be verified in situ. For example, the combination of DGGE with specific DNA probes has been used to reveal the presence of sulphate reducers in anaerobic sludge (Muyzer and Ramsing, 1995).

DNA fingerprinting techniques are ideally suited for high-throughput screening of biological reactors. With different sets of primers in PCR amplification, high specificity of the fingerprinting pattern can be obtained. Nevertheless, compared to FISH of whole cells it is subject to numerous biases during DNA extraction, amplification and cloning, and thus does not produce quantitative results.

4.2.2 16S rDNA sequencing

DNA sequencing and analysis of rRNA genes (16S rDNA) have revolutionized bacterial systematics. The most precise method of determining the phylogenetic affiliations of an isolate is sequencing of individual 16S rDNA molecules. The 16S rRNA genes code for the rRNA molecules needed for protein synthesis. The characterization of rRNA genes (rDNA) is a very powerful and frequently used means of accurately characterizing environmental isolates to phylogenetic levels ranging from domain (Bacteria, Archaea, and Eukarya) (Madigan et al., 2002) to a more specific phylogenetic placement. In comparison to phenotyping, 16S rDNA sequence analysis provides greater amount of genomic data that are directly linked to evolution and that can be used for phylogenetic comparison to all bacteria (Olsen and Woese, 1993).

16S rDNA sequences have been instrumental in classifying bacteria and in assigning their taxonomic position in phylogenetic trees. The entire 16S rDNA from a mixed population can be amplified by PCR and then cloned in a vector suitable for sequencing. In this way, organisms can be identified in a mixed population, even if the organism is non-cultivable. The selection of PCR primers determines which rRNA gene will be amplified. By combining non-specific PCR primers with cloning and sequence analysis techniques, it is possible to get information about the microbial sludge composition. For example, by applying PCR in combination with rRNA sequencing, a whole range of nitrifying-denitrifying microorganisms in activated sludge of an industrial WWTP was identified (Jurestchko et al., 2002). If selective primers are used, it is possible to amplify rRNA genes from specific groups of microorganisms present in the sludge. PCR amplification with methanogenic specific primers in combination with cloning and RFLP analysis was used to identify the methanogenic population in anaerobic sludge (Ng et al., 1994). PCR primers selective for ammonium oxidizing bacteria are also known (Voytek and Ward, 1994).

The advantage of 16S rDNA sequencing is that it gives an unambiguous identification of the organism in the community. The disadvantage of this protocol is that it is time-consuming and technically demanding.
4.2.3 Fluorescent in situ hybridization

In molecular biology, ribosomal RNAs are widely used for determination of evolutionary relationships among living organisms. 16S and 18S rRNAs originate from small (30s and 40s) subunit of the ribosome. Carl Woese at the University of Illinois pioneered the use of small subunit rRNA as a phylogenetic tool in the early 1970s, and the technique is now widely used. 16S rRNA based detection and identification methods have become very important in the unravelling of the microbial composition of wastewater treatment systems. One of the methods used for the analysis of the distribution of microbial population within a complex ecological setting is hybridization with rRNA based oligonucleotide probes (Amann, 1995; Stahl, 1995; Raskin et al., 1995), which are short single stranded DNA oligomers of 15–40 nucleotides that can be synthesized chemically. There are many reasons for targeting probes to 16S/18S of the small subunit of the ribosome or to the 23S/28S rRNA of the large subunit of the ribosome (Woese, 1987; Amann and Ludwig, 2000; Madigan et al., 2002):

- Ribosomes, and consequently rRNA molecules, are present in all organisms as an essential component of the protein synthesis apparatus and they have a homologous origin and show functional constancy,
- Some positions of the rRNA molecules are evolutionarily more conserved than others. Consequently, sequence regions can be found that allow differentiation at any taxonomic level from species and genera up to kingdoms or domains,
- The apparent lack of lateral gene transfer,
- Convenient lengths (about 1500 and 3000 nucleotides for 16S and 23S, respectively) with a range of very conserved to quite variable sites,
- The natural amplification of rRNA within microbial cells (usually more than 1000, frequently several 10,000 copies) makes it easier and more sensitive to assay this molecule and gives the opportunity for identification of single bacteria by fluorescent oligonucleotide hybridization,
- The presence and abundance of ribosomes and, consequently, rRNA in individual cells is connected to their viability and general metabolic potential,
- Availability of huge rRNA databases for comparative sequence analysis. The database of rRNA sequences in the Ribosomal Database Project (RDP) is now over 24000 (16000 aligned 16S sequences and 8000 aligned 18S sequences) (Madigan et al., 2002). The database can be accessed on the website: (http://www.cme.msu.edu/RDP/html/index.html) maintained by Michigan State University.

The technique is based on the concept that rRNA molecules, particularly the 16S and 23S rRNA molecules of prokaryotes are highly conserved through out evolution and are therefore useful as indicators of the phylogenetic affiliation of environmental isolates (Lane, 1991; Pettersen, 1997; Madigan et al., 2002). Two strategies usually are employed for rRNA analysis of isolated colonies: (1) colony hybridization with synthetic oligonucleotide probes detected toward specific phylogenetic groups and (2) analysis of the nucleotide sequence of the 16S rDNA. The first strategy can be used to quickly assign large numbers of isolates to relatively broad phylogenetic groupings. An extensive list of probes used for identification of a range of phylogenetic groups is presented by Amann et al. (1995) and Juretschko et al. (2002). Synthetic oligonucleotide probes are designed based on information available in databases such as the Ribosomal Data Base Project (RDP). Species-specific probes can be designed by targeting the most variable regions of the rRNA, whereas more general probes (group or genus) are complementary to the more conserved regions (Stahl and Amann, 1991).
The oligonucleotide probes are complementary to either variable or conserved regions of the rRNA. Undisturbed biofilms or flocs on gelatine-coated glass slides are treated with paraformaldehyde. The fixed and permeabilized cells are then exposed to DNA probe, often an oligonucleotide, which has been tagged with a fluorescent dye such as fluorescein. The DNA probe binds to complementary DNA or RNA sequences of the microbial cells that contain complementary sequences to produce signal when excited in an epifluorescence microscope (Figure 24). The probes can be applied after extraction of the rRNA from the sludge (dot-blot hybridization), but can also be used in situ in combination with fluorescent microscopy.

The applications of rRNA-based nucleic acid techniques to the analysis of wastewater treatment systems today range from a simple identification of isolates over the detection of bacterial diversity and population dynamics to fully and quantitatively describing the complex microbial communities. Ribosomal RNA-targeted oligonucleotide probes are ideally suited to investigate the composition of complex microbial communities. By targeting more conserved sites of the rRNA, the domain- and group-specific oligonucleotide probes can discriminate between the three domains Archea, Bacteria and Eucarya (Amann et al., 1995), or identify members of larger phylogenetic groups such as alpha-, beta-, and gamma-subclasses of Proteobacteria (Manz et al., 1992), the Cytophaga-Flavobacterium cluster (Manz et al., 1996), Gram-positive bacteria with a high (Roller et al., 1994) or low (Meier et al., 1999) DNA G+C content, or planctomycetes (Neef et al., 1998). Group-specific probing yields important data on the abundance of different phylogenetic groups in different environments.

Both group- and species-specific 16S rRNA-targeted oligonucleotide probes have also successfully been used for the investigation of the microbial community composition of a nitrifying–denitrifying activated sludge from an industrial sewage treatment (Jurestchko et al., 2002), population analysis of Paracoccus spp. in biofilm (Neef et al., 1996), for in situ identification and phylogenetic analysis of activated sludge bacteria (Snaider et al., 1997) and study of the nitrifying population dynamics (Mobarri et al., 1996; Schramm et al., 1996; Jurestchko et al., 1998; Schramm et al., 1998; 1999; Daims et al., 2001; Gieske et al., 2001, Egli et al., 2003; Nogueira et al., 2002).

Another important development is the combination of bacterial community composition analysis by rRNA probing with in situ activity measurements. Recent studies have combined FISH with microsensors (Ramsing et al., 1993; Schramm et al., 1996, 1998, 1999; Okabe et al., 1999), microautography (Lee et al., 1999; Overney and Fuhrman, 1999) and substrate uptake rate measurements (paper IV). FISH in combination with in situ activity measurements would significantly widen our understanding of the microbial interactions in a given ecological setting. Moreover, 16S rRNA-targeted oligonucleotide probes have been found to be useful in bio-augmentation research for the identification and monitoring of introduced specialized microorganisms in complex microbial communities in polluted sites and bioreactors. FISH techniques have successfully been used to study and monitor the introduced nitrifying bacteria in biofilm (Satoh et al., 2003), population dynamics of the introduced Pseudomonas spp. for the degradation of chlorinated benzenes (Tchelet et al., 1999; Wenderoth et al., 2003), Microvirula aerodenitrificans in a seeded batch reactor (Patureau et al., 2001), and enhanced nitrogen removal by bio-augmentation with Brachymonas denitrificans CCUG 45880 (paper IV).

The combined use of different fluorescent dyes offers very interesting possibilities to study the ecology of certain strains within a community of related organisms. By using oligonucleotide probes labelled with different dyes with different excitation wavelengths (carboxyfluorescein, 488nm; tetramethylrhodamine, 543 nm; and Cy5, 633 nm), seven distinct
genotypes of closely related bacteria in activated sludge were visualized (Amann, et al., 1996). The presence or absence of particular genetic sequences within culturable isolates of a microbial community can be determined by hybridization of the isolated colonies with gene probes or cloned versions of the genes of interest (Staley et al., 1985). Probes can be directed toward specific functions, such as enzymes encoding the degradation of specific pollutant, or toward specific phylogenetic groups.

Some of the limitations of FISH technology are: 1) the 16S rRNA may be too well conserved to discriminate between closely related populations (Fox et al., 1992). In such cases, the 23S rRNA with highly variable regions may be useful. 2) Current knowledge of the rRNA diversity is still incomplete (Amann et al., 1995). The ability to quantify populations in complex microbial communities is directly correlated with the size and quality of the rRNA databases and the continuous development of tools for probe design and in silico specificity control (Amann and Ludwig, 2000). 3) The most important practical limitation for the wide application of rRNA-targeted nucleic acid probes in microbial ecology is the lack of automation. Currently, only a limited number of samples can be processed with a restricted set of probes (Amann and Ludwig, 2000). 4) In certain circumstances where the rRNA content of cells may be below detection limit or the cell envelopes are impermeable to fluorescently labelled probes, FISH cannot give full picture of the microbial distribution.

With the development of microarray/DNA chip technology (Peples et al., 2003; Zhou, 2003) environmental samples can be probed with several kinds of probes in a single reverse hybridization. Microarrays are a recently developed, powerful genomic technology that is widely used to monitor gene expression under different cell growth conditions, detect specific mutations in DNA sequences and characterize microorganisms in environmental samples (Zhou, 2003). However, the specific hybridization of target molecules to immobilized capture oligonucleotides is one of the major challenges of the DNA microarray approach because large sets of probes with different characteristics are applied under identical hybridization conditions (Peples et al., 2003).

In general, recent advances in molecular biology have the potential to enhance environmental biotechnology. The ability to characterize microorganisms rapidly in mixed culture on a phylogenetic basis provides a way of monitoring community structure and biodiversity in natural and engineered systems. Applying molecular techniques to the detection and quantification of structural genes holds the promise of rapid and sensitive determination of microbial activities. One pivotal concern with all of these is specificity. Although the rDNA sequence database has grown rapidly, the number of sequences available is still small compared to the number of different bacteria in the environment. Care must be taken in designing probes and primers, choosing sufficiently stringent hybridization conditions, and in the interpretation of results. Moving these rapidly evolving molecular techniques into mainstream industrial use is a pressing challenge that requires the protocols to be clear, dependable and affordable. The prospects of environmental biotechnology in wastewater management are quite immense and their wide application with practical impact is certain to grow in the years to come.
5 PRESENT INVESTIGATIONS

The results of the research are reported in five papers referred to, in the text, by the Roman numerals (I-V). The study was focussed on (i) characterization of tannery effluents and assessment of downstream pollution profiles to map out the extent of environmental impact of the tanning industry in Ethiopia (paper I), (ii) isolation and characterization of the efficient denitrifying microorganisms and study their denitrification potential in tannery wastewaters laden with toxic substances (paper II, IV), (iii) development of bioprocesses and evaluation of their effectiveness for biological nitrogen and organic matter removal from tannery wastewaters (paper III), (iv) bio-augmentation of a pilot process plant with the active denitrifying bacterial isolates and evaluate whether this approach can be used to enhance biological nitrogen removal from tannery effluents (paper IV), and (v) study the potential of the predenitrification-nitrification process for biological sulphide removal from tannery effluents (paper V).

5.1 Characteristics of tannery effluents and downstream pollution profiles (paper I)

In paper I, the salient features of tannery effluents were characterized and the deleterious environmental effects of various pollutants were described. In order to determine the potential environmental impacts of tanneries, Modjo tannery, located 70km from the Capital Addis Ababa, Ethiopia, was selected as a case study. Modjo tannery is a medium sized tanning facility with installed capacity of processing 844,000 and 1,656,000 sheep and goatskins, respectively, per annum (EPA, 2002; Table 1). The plant is sited near the Modjo river and channels its effluents directly to the river (Figure 6). The volume of wastewater discharge into the Modjo river varies between 3500-5500 m³day⁻¹. As shown in Figure 3, the river drains into Lake Koka after some 25km and the river water is used for irrigation, fishing, and swimming purposes along the entire downstream length. In addition to Modjo and Ethiopian tanneries, there are other two tanneries operating along the river, namely Shewa tannery and Molba slaughter and tannery, as located on the map in Figure 3 by S₂ and S₃, respectively. The last two tanneries are newly constructed and unless a proper waste management system is taken care of it will further exacerbate the pollution of the river as well as the reservoir lake, Lake Koka.

In this study, a detailed wastewater characterization was performed using standard methods for a number of selected parameters that are considered to be deleterious on the receiving environment. COD, total N, ortho-phosphorus, ammonia N, sulphides and sulphates were all measured colorimetrically (Dr2010, USA), BOD₅ and total chromium was determined according to standard methods (APHA, 1992). TDS, conductivity and salinity of the wastewaters were measured using conductivity metre (CO 150, USA). Temperature and pH of the samples were measured using portable pH metre.
The potential environmental impacts of Modjo tannery wastewater effluents are high. Composite untreated wastewater effluents from Modjo tannery is turbid, coloured and foul smelling (Figure 7). It consists of acidic and alkaline liquors, with chromium levels of 10–65 mg/l, sulphide levels of 300–900 mg/l, nitrogen levels of 600–2500 mg/l, Ammonia nitrogen levels of 100–150 mg/l, COD levels of 8000–15000 mg/l, and BOD₅ ranging from 2000–5000 mg/l (Table 6, paper 1). Significant volumes of solid wastes are produced, including trimmings, degraded hide, and hair from the beam house processes (Figure 8). Decaying organic material produces strong odours. Hydrogen sulphide is released during dehairing, and ammonia is released in deliming (Figure 2).
Figure 7. Showing composite untreated tannery effluents

Figure 8. Solid waste pileups from Modjo tannery
Table 6. Characteristics of Modjo tannery effluent wastewaters
(Concentrations are in mg/l except for pH, temperature, conductivity and salinity)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>10.8±0.08</td>
<td>10.5 – 11.3</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>21.2±0.8</td>
<td>18.3 – 24.0</td>
</tr>
<tr>
<td>COD</td>
<td>11123.0±563.9</td>
<td>7950 – 15240</td>
</tr>
<tr>
<td>BOD₅</td>
<td>2982.5±259.0</td>
<td>1918 – 4640</td>
</tr>
<tr>
<td>Ammonia N</td>
<td>122.2±8.3</td>
<td>81 – 164</td>
</tr>
<tr>
<td>Total Nitrogen</td>
<td>1330.0±182.1</td>
<td>680 – 2640</td>
</tr>
<tr>
<td>Ortho-phosphorus</td>
<td>15.5±7.0</td>
<td>4 – 27.2</td>
</tr>
<tr>
<td>Sulphides</td>
<td>630.4±67.0</td>
<td>325 – 932.5</td>
</tr>
<tr>
<td>Sulphates</td>
<td>502.0±82.9</td>
<td>280 – 1180</td>
</tr>
<tr>
<td>Total chromium</td>
<td>32.2±5.7</td>
<td>12.3 – 64</td>
</tr>
<tr>
<td>Total dissolved solids</td>
<td>6646.0±556.5</td>
<td>4480 – 9660</td>
</tr>
<tr>
<td>Conductivity (µS/cm)</td>
<td>14330.0±182.3</td>
<td>9500 – 20060</td>
</tr>
<tr>
<td>Salinity (%)</td>
<td>7.1±0.6</td>
<td>6 – 10</td>
</tr>
</tbody>
</table>

As shown in Table 6 above, the leather industry generates a strong wastewater with high 3OD₅, COD, total N, sulphide and TDS concentrations. In addition to these pollutants, trivalent chromium is the major pollutant of concern. This wastewater is ultimately discharged into adjacent natural water bodies and cultivated lands. The discharge, of highly toxic tannery wastewater, high in BOD, COD, TDS, sulphide, chloride and chromium, has rendered the Modjo river water unfit for irrigation, recreational and livestock consumption purposes. This quality, as well as quantity deviation from NEQS set by the EPA implies discharge of huge quantities of toxic wastewater into the water environment, which can have severe environmental and public health effects (Figure 9).

As shown in Figure 10, the downstream pollution profiles along the river course varied from 45 to 475 mg/l for COD and 15 to 165 mg/l for BOD₅ indicating high organic pollution of the Modjo river down stream. Similarly, total N and ammonia N concentrations were higher (35–100 mg/l and 5–50 mg/l, respectively). As a result eutrophication in the river, especially in the reservoir Lake Koka is likely to occur; this could adversely affect the use of the river as well as the lake water for recreational and other purposes. Chromium and sulphide were detected in higher levels (2–15 mg/l and 2–30 mg/l, respectively) in all the downstream river samples, indicating that pollution from these chemicals was due to discharges from tannery effluents in the area. Chromium was not detected in upstream samples and sulphide was detected in small amounts (Figure 10). Ethiopia’s discharge limit values for total chromium and sulphide in water bodies are 2 mg/l and 1 mg/l, respectively (EPA, 203). Owing to their potential toxicological effects, discharge of these substances is normally subjected to stringent regulations in many countries (Bosnic et al., 2000).
Figure 9. Modjo river downstream users
Figure 10. Modjo downstream river pollution profiles for the four sampling sites along the Modjo river. Note: S stands for sampling sites and Su for upstream sampling site.

The downstream pollution profiles presented in paper I provides a picture of trends of pollution of water bodies by tanning industries and the urgent need for measures to bring tanning industries to acceptable environmental performance in the country. It can also be used as a basis while defining limits for discharge of tanning operations in the country. These facts must regularly be brought to public awareness in developing countries like Ethiopia where industries in general and tanneries in particular, discharge their wastes directly into the environment without considering the ecological consequences.

5.2 Identification and characterization of the six most efficient denitrifying bacteria from tannery effluents (paper II)

Denitrifying microorganisms have been isolated from a number of habitats (Schocker et al., 1991; Fries et al., 1994; Zhou et al., 1995) belonging taxonomically to the various subclasses of the Proteobacteria and encompass a wide range of physiological traits (Zumft, 1997). Not all of the microorganisms in an ecological system will be crucial for deriving the desired biochemical transformations. In this study, six isolates from over 1000 pure cultures were characterized and identified as having extraordinary denitrifying properties in tannery effluents laden with toxic substances such as chromium and sulphides (paper II). The focus of this study was to identify denitrifying bacteria that possess remarkable ability to denitrify and tolerate the unique environments of tannery effluents.

5.2.1 Selection of potential denitrifying bacteria from tannery wastewaters

In order to identify potential microorganisms with denitrifying properties, more than 1000 pure cultures of bacteria were isolated from Modjo tannery pilot and Ethiopia tannery WWTPs. Isolations were made aerobically on nutrient agar (Difco) and incubated for two days at 30°C and 37°C. Denitrifying organisms were identified as gas-producing bacteria when grown anaerobically in nutrient broth (Difco) supplemented with 0.15% NaNO₃ and 0.1% agar and incubated at 30°C (Gerhardt et al. 1981). Denitrifying activity was measured as
NO₃⁻ uptake over time in microtitre plates using the method described in Gumaelius et al. (1996, 2001). In the determination of denitrification rates of the isolates, nitrite reduction was chosen for three reasons: (i) it is the defining reaction of denitrification, the step that differentiates denitrifiers from nitrate respirers; (ii) the denitrification rate is measured as an activity of the enzyme nitrite reductase (Zumft, 1997; Shapleigh, 1999); (iii) the process is relatively easy to analyse and the analysis itself is fast.

Depending on their denitrification status, 28 isolates were selected from 1000 bacterial isolates. Figure 11 shows the denitrification rates for all the 28 isolates including the reference strain *Brachymonas denitrificans* JCM 9216.

![Figure 11. Denitrification rates for the 28 examined denitrifying isolates and *Brachymonas denitrificans* JCM 9216. Rates were as an average values where n = 2.](image)

### 5.2.2 Biochemical characterization of the denitrifying isolates

The selected 28 isolates of denitrifying bacteria were evaluated using their reaction kinetics in the Phene Plate™ (PhP-48) system (Phene Plate AB, Stockholm). The Phene Plate system is a miniaturised metabolic fingerprinting system designed to identify bacterial strains at subspecies level.

Fresh cultures of bacterial strains cultivated on nutrient agar were suspended into a medium containing 0.01% proteose peptone (Oxoid) and 0.011% bromothymole blue indicator. Aliquots of 0.150 ml of the bacterial suspension were added, using multichannel pipette, to each of the PhP-48 wells of microtitre plates containing the dehydrated biochemical substrates. Two strains were analyzed per plate and the plates were incubated at 30°C and A₆20 of each well was read after 16, 40 and 64 hours with a fully automated microprocessor controlled microtitre plate reader (Sunrise Tecan, Ges.m.b.H, Austria). All data manipulation including conversion of absorbance values to PhP data, correlation coefficients, diversity indices, and population similarities, clustering and printing of dendrograms was performed using PHPWIN software, version 2.1 (PHP Plate AB, Stockholm). A dendrogram was constructed to reflect these similarities using the clustering method of unweighted-pair group method with mathematical averages (UPGMA) with identity level set as 0.98. Isolates were assigned to the same biochemical phenotype (BPT) if they clustered together at a similarity level of 0.98 and above. A common phenotype was defined as one comprising two or more strains, whilst single strains were referred to as single phenotype (Kühn et al., 1991, 1997).
As shown in paper II, the phenotypic diversity of the 28 denitrifying strains isolated from the two tannery wastewater treatment systems were identified as three different phylogenetic groups plus seven different single phenotypes based on their biochemical fingerprints (Figure 12).

![Dendrogram showing clustering relationships of denitrifying strains isolated from tannery wastewaters obtained from biochemical fingerprinting using UPGMA clustering method.]

**Figure 12.** Dendrogram showing clustering relationships of denitrifying strains isolated from tannery wastewaters obtained from biochemical fingerprinting using UPGMA clustering method.

The two phylogenetic groups, BPT1 and BPT2 containing 4 and 11 isolates, respectively, and the seven singles were from Modjo tannery pilot WWTP whereas the third phylogenetic group, BPT3, was from the Ethiopia tannery WWTP (Figure 12). Even though these two treatment plants are similar with regard to the nature and composition of their wastewaters (paper I, III), one dominant group of denitrifying population was observed in Modjo while different denitrifying species were found in Ethiopia tannery WWTP. The relatively short life (three years) and the fluctuation in influent feeding of wastewaters at Modjo pilot WWTP might account for the observed differences in the denitrifying populations in the two systems.

One can also infer from the result that only a narrow range of denitrifying species was able to grow in the Ethiopia tannery wastewater environment due to the toxic nature of the wastewater. However, culture dependent methods may underestimate the true picture of the composition of microorganisms in complex microbial communities (Amann et al., 1995). On the other hand, the low diversity and highly selective nature of the tannery wastewater environment might be an encouraging phenomenon, in terms of enriching a consortium for effective denitrification of tannery wastewaters (paper IV).
Figure 13. Diversity of denitrifying populations obtained from biochemical fingerprints: BPT = biochemical phenotypes containing more than one similar strains; PTSi = phenotypes containing single strains as shown in Figure 12.

5.2.3 Characteristics of denitrification and determination of the nitrite-reductase type enzyme of the efficient denitrifying strains

The BPT3 phylogenetic group (paper II) that was isolated from the Ethiopia tannery WWTP was shown to have extraordinary denitrifying properties from all the isolates (Figure 11). The denitrification rate for this phylogenetic group ranged between 0.47 and 0.59 mg NO₂⁻ ¹ h⁻¹. This denitrifying activity was similar to the values reported for Comamonas denitrificans, another efficient denitrifying bacterium, by Gummelius et al. (2001). From the amplification of nitrite reductase gene, all the BPT3 strains also possess the cd₁-type of nitrite reductase (Table 7).

Genomic DNA was obtained from pure cultures of the strains by protease K, sodium dodecyl sulphate (SDS) and hexadecyltrimethylammonium (CTAB) treatments, followed by phenol-chloroform extraction and subsequent precipitation with isopropanol using the method as described in Ausubel et al. (1991). For determination of nitrite reductase type a PCR based method developed by Braker et al. (1998) was used. Primers used were nirS1F and nirS6R for detection of the cd₁-type nitrite reductase and nirK1F and nirK5R for the Cu-type nitrite reductase. PCR reactions were performed in a total volume of 50 µl containing 5 pmol of each primer and 100 ng of DNA. A “touchdown” PCR was performed with the annealing temperature varied between 40°C and 45°C. The amplification products were electrophoresed on 2% (w/v) agarose gel (Sigma-Aldrich, USA) containing 0.5 mg litre⁻¹ ethidium bromide. Pseudomonas stutzeri (ATCC 14405) and Alcaligenes faecalis (ATCC 8750) were used as positive controls for Cd₂-Nir and Cu-Nir types, respectively.

Nitrite reductase is the enzyme that catalyses the nitrite reduction to nitrous oxide (eq. 4). This enzyme is found as two variants. One contains copper as redox active metal and is encoded by NirK, while the other contains hemes c and d₃ and is encoded by NirS (Braker et al., 2000). Even though there is no clear phylogenetic distribution of the two enzymes, both types can be found within members of a single genus but they are mutually exclusive in a single species (Coyne et al., 1989). The cd₁-type was found to be more abundant in nature (Coyne et al., 1989; Zumft, 1992).
5.2.4 Cellular fatty acid profiles and molecular characterization of the efficient denitrifying strains

Gas chromatographic analyses of whole-cell fatty acids showed that all the six denitrifying organisms appeared to have similar fatty acid compositions (paper II). The cellular fatty acid profiles of this phylogenetic group indicated that they were all similar to each other and to Brachymonas denitrificans, representative of the β-subclass of the Proteobacteria. One of the six efficient denitrifying strains (B797 CCUG 45880) was further analysed by 16S rRNA partial sequence analysis, and phylogenetic affiliations were elucidated by comparison of the sequences from the databases from the Genbank, EMBL, DDJB, and PDB. Strain B797 (CCUG 45880) was found to be identical to Brachymonas denitrificans JCM 9216 (D14320), a representative of the β-subclass of the Proteobacteria, with a sequence similarity of 99% confirming the genetic coherency of the isolates as a single species. The reference strain (Brachymonas denitrificans JCM 9216) was isolated from activated sludge in Japan and is known to be aerobic chemoorganotrophic rhodoquinone-containing denitrifying bacterial species (Hirashi et al., 1995).

5.2.5 Determination of the effects of chromium (III) and sulphide on the efficient denitrifying strains

In order to determine the inhibitory effects of chromium and sulphide, known toxicants present in tannery wastewaters, on denitrification rates, and their relationship to nitrogen removal processes in the treatment of tannery wastewaters, the efficient strains were used as biomarkers for denitrification inhibition (paper II). Chromium (III) and sulphide were added to final concentrations ranging from 30 to 70 mg/l and 75 to 150 mg/l, respectively. In both cases, 5 μl samples were taken every 30 min during incubation and nitrite reduction as a measure of denitrification was assessed. Inhibition was calculated as percentage inhibition compared to a reference sample containing no toxicant.

As shown in Figure 14, the decrease in denitrification rate of the strains was linearly proportional to an increase in chromium (III) and sulphide concentrations. For both toxicants similar patterns of inhibition were observed for the six strains. Interestingly, the efficient denitrifying strains were 50% inhibited by chromium (III) and sulphide at concentrations of 54 and 96 mg/l, respectively, whereas 50% inhibitions to the reference strain Brachymonas denitrificans JCM 9216 was seen at a concentration of 25 mg chromium (III)/l. In this study, significant inhibitory effects of the two toxicants on denitrification rates of the strains was shown at concentrations of 70 and 150 mg/l, respectively.

The denitrification rates of the efficient denitrifying strains in the presence of high toxicant concentrations in this study showed that the six strains not only had high nitrogen removal capability from tannery wastewaters but also possessed high tolerance properties to these substances in such environments. A typical untreated tannery wastewater contains 70 mg chromium (III)/l and 160 mg sulphide/l, UNEP, 1991; paper I, III). The fact that similar patterns of denitrification were observed with that of the reference strain while having higher tolerance to the toxicants supported the hypothesis that the efficient denitrifying isolates have become tolerant to toxic environments of tannery wastewaters. These properties make the application of the strains more reliable and feasible in bioremediation processes of industrial wastewaters such as tannery that is characterized by high content of nitrogenous compounds, chromium and sulphide (paper I, III). The salient key features of the efficient denitrifying bacteria identified in this study are given in Table 7.
Table 7. The six selected efficient denitrifying strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nucleotide Accession No.</th>
<th>Phylogenetic affiliation</th>
<th>Some key properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>B79^T</td>
<td>CCUG 45880</td>
<td></td>
<td>• possess nirS genes</td>
</tr>
<tr>
<td>B11</td>
<td>CCUG 45881</td>
<td></td>
<td>• excellent denitrifying ability</td>
</tr>
<tr>
<td>B12</td>
<td>CCUG 45882</td>
<td>Brachymonas denitrificans</td>
<td>• high tolerance capacity to toxicants</td>
</tr>
<tr>
<td>B15</td>
<td>CCUG 45883</td>
<td></td>
<td>• biomarkers for denitrification inhibition</td>
</tr>
<tr>
<td>B28</td>
<td>CCUG 45884</td>
<td></td>
<td>• Ecologically useful group in tannery WWT systems</td>
</tr>
<tr>
<td>B38</td>
<td>CCUG 45885</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.3. Development of processes for biological nitrogen and organic matter removal from tannery wastewaters (paper III)

5.3.1. A pilot process plant

In this study, a pilot plant consisting of a predenitrification anoxic system, aerated nitrification compartment and a sedimentation tank (clarifier) all arranged in series as shown in Figure 15 was developed and installed on the premises of Addis Ababa University, Ethiopia. A view of the pilot process plant is shown in Figure 16. The anoxic denitrification tank, the aerated nitrification reactor and the clarifier have a volume of 100, 200 and 50 litres, respectively. The influent wastewater was pumped at a rate of 5 l h^-1 and the hydraulic residence time (HRT) of the anoxic and aerobic reactors were maintained at 20 and 40 hours, respectively. The MLSS from the activated sludge and the sludge from the effluent were recycled to the anoxic denitrification tank by a peristaltic pump and airlift pump, respectively.
In this system, both sludge and mixed liquor are recycled. Sludge recycling is to maintain a desired level of MLVSS concentration and mixed liquor recycling is required to produce a final effluent, which has a low residual nitrate concentration. The anoxic zone is the main denitrification reactor in the process. The absence of oxygen and the presence of nitrate or nitrite lead to the enrichment of denitrifying bacteria, which reduce nitrate/nitrite to molecular nitrogen. Soluble and colloidal biodegradable matters are readily removed in the anoxic zone.

Two principal biochemical processes are possible under this anoxic environment:
- anoxic organotrophic oxidation of organic compounds (when nitrate nitrogen as an electron acceptor is reduced to nitrogen gas, the process is termed denitrification)
- anoxic chemolithotrophic oxidation of sulphide and elemental sulphur to sulphate (paper V).

The most important biochemical processes in oxic conditions are:
- oxidation of organic compounds (organotrophic metabolism)
- chemolithotrophic oxidation of ammonia and nitrite to nitrate (nitrification) and of reduced sulphur compounds to sulphate

5.3.2 Start-up of the process

The raw tannery effluent lacks the necessary microbial populations for the biological degradation of the wastes. One strategy to improve a particular aspect of process performance in WWTP, for example during start-up, is the addition of specialized microorganisms or activated sludge from another WWTP (Rittmann and Whiteman, 1994). The anoxic and nitrification reactors of the pilot plant were initially seeded with a mixture of (90:10) acclimatized activated sludge inoculum consisting of bacterial flora adapted to tannery waste from the Ethiopia tannery WWTP and from the Kaliti pond system, which treats municipal wastes. The system was subsequently fed with combined raw tannery wastewaters (soaking, pickling and tanning processes) that were sampled from the Modjo tannery. The feeding process consisted of four phases with 30, 28, 26 and 33 days each, respectively (Table 8). The influent and effluent wastewater samples were routinely analyzed for various parameters as described above (paper III).
Figure 16. View of the pilot process plant installed at AAU, Ethiopia (this study)
Table 8. Pilot plant operational parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Feeds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>COD overall loading rate (Kg.m⁻³ day⁻¹)</td>
<td>1.13</td>
</tr>
<tr>
<td>BOD₅ overall loading rate (Kg.m⁻³ day⁻¹)</td>
<td>0.3</td>
</tr>
<tr>
<td>MLSS recycle, R</td>
<td>1R</td>
</tr>
<tr>
<td>Sludge return, R</td>
<td>1R</td>
</tr>
<tr>
<td>MLSS (g/l)</td>
<td>2.4</td>
</tr>
<tr>
<td>MLVSS (g/l)</td>
<td>1.7</td>
</tr>
<tr>
<td>SVI (ml/g)</td>
<td>38.0</td>
</tr>
<tr>
<td>DO aerated (mg/l)</td>
<td>2.2</td>
</tr>
<tr>
<td>DO anoxic (mg/l)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

5.3.3 The pilot process plant performance efficiency

The influent tannery wastewaters were characterized by high alkalinity, with a resulting pH value of above 10.0 due to the chemicals used in the leather processing (paper I, III). Despite high influent perturbations over the successive feeds, the effluent parameters obtained for the various pollutant concentrations were satisfactory (Figure 17). Specifically, the effluent total N, ammonium N, COD, BOD₅, sulphide and chromium (III) concentrations registered in the fourth experimental feed were in line with the discharge limit values adopted for tannery effluents in to surface water bodies in many countries (Bosnic et al., 2000; EPA, 2003).

![Figure 17](image-url)  
**Figure 17.** Influent and effluent concentrations of the pilot plant for the six months study period.
Despite high chromium and sulphide contents in the influent, high removal efficiencies were obtained for total N and COD ranging from 82–98% and 95–98%, respectively for the experimental feeds. Higher COD removal in the pilot plant processes was associated with higher denitrification efficiency of the system (Figure 18). BOD₅, sulphide and chromium (III) were nearly almost completely removed during all the runs (paper III, V). Ammonium N removal efficiency of the system slowly increased from 46–95% and was highest at a COD loading rate of 0.72 kg m⁻³ day⁻¹.

Figure 18. Treatment efficiencies of the pilot process plant over the study period.

The successful operation of activated sludge processes treating tannery effluent involves the management of abundant and active populations of organotrophs (mainly denitrifying and nitrifying bacteria). Proper interactions between organotrophs/chemorganotrophs and nitrifying bacteria are required to remove organic matter, (cBOD) and nitrogenous wastes (nBOD). By using organic wastes as a substrate for carbon and energy, organotrophs reduce the quantity of cBOD in the activated sludge process. By using carbon dioxide as carbon and ammonium and nitrite as energy substrates, nitrifying bacteria decrease alkalinity in the nitrification tank and reduce the quantity of nBOD in the activated sludge process. The lower organic loading rates also result in decreased oxygen transfer requirements for the aerated basin (paper III, Cloete and Muyima 1997, Cheremisinoff, 1996, Gerardi, 2002).

Good performance depends on the right choice of organic loading rate in order to ensure proper floc formation and obtain more than 90% removal efficiencies (Gerardi, 2002, paper III). As can be seen from Figure 18, ammonium N removal efficiency was affected by the influent loading perturbations during the experimental feeding phases. As the COD loading rate decreased from 1.1 to 0.7 kg m⁻³ day⁻¹, ammonia N removal efficiency of the system increased from 46% to 95% (Figure 19).

Since nitrifying bacteria are slow growers and acclimatize over time with increasing population size (Cloete and Muyima, 1997; Bitton, 1999; Gerardi, 2002), an experiment was conducted after a year with increased organic loading rate in order to evaluate whether the system could possibly withstand such loading perturbations. As shown in Figure 19, at a COD loading rate of 1.5 kg m⁻³ day⁻¹, ammonium N removal rate increased to 70% and as organic loading rate increased to 2.0 kg m⁻³ day⁻¹, the corresponding ammonium removal rate was only 40%. The result showed that ammonia oxidation was slightly increased by 10% when COD loading rate was increased by 50% of the original loading rate. As the loading rate increased by 100%, the removal rate again decreased verifying that high organic loading to the aeration
tank has affected the nitrification efficiency of the system. Higher organic loads accelerate the heterotrophic growth rate and the sludge quantities produced in the system thus decreasing the fraction of nitrifiers present in an activated sludge system (Kristensen et al., 1992; Gupta and Gupta, 2001; Jokela et al., 2002, Gerardi, 2002). In this case, the contribution from the autotrophic nitrifying bacteria would be minimal.

![Figure 19](image)

*Figure 19.* The effect of organic loading rate on the ammonium removal efficiency of the pilot plant. Note: The first four feeding experiments were carried out from March to August 2002. After a year, the experiment was conducted with increasing loading rate to check whether the system could withstand influent loading perturbations overtime.

Similarly, a lower DO level in the aeration tank was observed to decrease the ammonium N removal rate of the system (Figure 20). Of the many operational requirements known to affect nitrifying bacteria, dissolved oxygen (DO) concentration is one of the most important requirements. Because nitrifying bacteria are strict aerobes, they can only nitrify in the presence of dissolved oxygen. At DO concentrations less than 2 mg/l, the nitrification rate of the treatment plant would be low, and at DO < 0.5 mg/l, almost no nitrification occurs (Gerardi, 2002). With increasing DO concentrations, nitrification accelerates (Figure 20). Because nitrifying bacteria must reduce CO₂ for growth and obtain little energy from ammonia and nitrite, there is a competition for DO with organotrophs in the activated sludge system. Therefore, the DO level within the aeration tank should be carefully monitored and kept above 1.5 mg/l (Cheremisinoff, 1996; Gerardi, 2002).

Nitrification, like most bacterial processes, is also affected by pH and temperature. Studies show that the optimal range of pH and temperature for nitrification is 6.5–8.6 and 5–30°C, respectively (Cheremisinoff, 1996; Grunditz et al. 1998; Im et al., 2001; Gerardi, 2002). In this study, the pH and temperature of the nitrification reactor varied from 7.5–8.2 and 15–20°C, respectively, indicating that these parameters were in the normal range of operating conditions in the system.
Alkalinity is lost in an activated sludge process during nitrification. This loss occurs through the use of alkalinity as carbon source by nitrifying bacteria and the destruction of alkalinity by the production of hydrogen ion (H⁺) and nitrite ions during nitrification (eq.5). Significant alkalinity is lost through oxidation of ammonia and this result in a decrease in pH below to 6.7, which significantly decreases the nitrification rate. Tannery wastewater is normally alkaline. The alkalinity lost during nitrification was compensated from the pre-denitrifying reactor.

\[ \text{NH}_4^+ + 1.5\text{O}_2 \rightarrow \text{Ammonia oxidizing bacteria} \rightarrow 2\text{H}^+ + \text{NO}_2^- + 2\text{H}_2\text{O} \] (5)

The process involved in nitrogen removal from tannery effluents must be not only technically efficient but also must be able to meet the effluent criteria in an economically viable manner. The activated sludge reactors with pre-anoxic tank employed for high strength wastewater in this study were not affected by shock loads and could achieve acceptable effluent quality in spite of dynamic influent feeds (paper III, IV, V), although they do perform better under more stable environments.

5.3.4 **Determination of denitrification potential of the pilot plant**

The biomass of the activated sludge is the active agent in biological wastewater treatment. To describe and control the processes occurring in WWTPs, this biomass is often characterized in terms of: 1) characterization and quantification according of metabolic activities, 2) identification and classification of microorganisms (paper II), 3) activated sludge quality. The most common methods of characterization and quantification of activated metabolic activities are indirect, i.e. the rate of consumption of components entering the biochemical reactions or the rate of formation of reaction products is measured instead of quantifying specific metabolic groups (Cloete and Muyima, 1997). Thus the nitrifying and denitrifying capacity of
activated sludge is determined by nitrification rate (measured as Ammonia Uptake Rate (AUR) or nitrate production rate), and denitrification rate (Nitrate Uptake Rate, NUR).

Some of the important applications of NUR as a tool for wastewater characterization include: 1) establishment of the denitrification activity for a given sludge, 2) determination of the sludge capacity for anoxic degradation of wastewater or a specific carbon source, 3) determination of the fraction of denitrifying bacteria in a specific activated sludge, and 4) quantification of the quality of a given wastewater or a specific carbon source for denitrification (Kristensen et al., 1992).

In order to determine the denitrification capacity of the pilot plant system, NUR batch experiments of the sludge from the pilot plant were determined in laboratory scale reactors. Two 2-l capacity lab scale reactors were used where 1.5 l activated sludge was added to each reactor. In the first reactor, tannery wastewater was used as internal carbon source and the second reactor was used as a reference where acetate (normally a very easily degradable carbon source under anoxic conditions) was added. The NUR experiments were carried out under anoxic conditions by flushing the reactors with N2 gas. NO3-N was added to both reactors at 60 mg/l to be removed and the reduction rate is monitored through sampling and analysis. NUR was calculated as a change of nitrate nitrogen (mg NO3-N gVSS\(^{-1}\)h\(^{-1}\)). The basic principle of the test as shown in Figure 21 is further described in Kristensen et al. (1992).

**Figure 21.** Illustrations of the principle of NUR determination.

The NUR experiments gave results varying from 6.5–9.3 mg NO3-N gVSS\(^{-1}\)h\(^{-1}\) for the test wastewater used as an internal carbon source and 5.7–7.7 mg NO3-N gVSS\(^{-1}\)h\(^{-1}\) for the reference (Figure 21). The higher denitrification rate for the tannery wastewater as internal carbon source than acetate indicates that tannery wastewaters contain broader and more easily biodegradable organic matter that can maintain more denitrifying consortia to be active in the system. It also shows a complete denitrification capacity of the system without the need for any external carbon source. The information about the relation between the sludge loading rate and the obtainable denitrification rate for external carbon source is of interest in processes using external carbon sources to enhance denitrification. Various reports (Kristensen et al., 1992; Kajuwa and Klapwijk, 2000) show that the nitrate utilization rates have effectively been used to determine the denitrification and nitrification capability of a treatment system.
5.3.5 Effect of chromium (III) on AUR

The inhibitory effect of chromium (III) on the nitrification activities of the sludge from the pilot plant was determined on AUR batch experiments using laboratory scale reactors. Chromium (III) was added at concentrations of 50, 85, 170, and 250 mg/l to the first reactor and the second reactor was used as a reference for calculating the actual nitrification rate of the sludge. Toxicity was quantified in terms of the reduction in AUR of the microorganisms in the activated sludge versus that in the references. AUR was calculated as mg NH₄-N g VSS⁻¹ h⁻¹. The AUR test was described in detail in Kristensen et al. (1992). The nitrification rate without chromium (III) varied from 4.3–6.4 mg NH₄-N g VSS⁻¹ h⁻¹ while AUR was decreasing with increasing toxicant concentrations (Figure 23).

AUR investigations with different concentrations of chromium (III) showed 50 % inhibition at concentrations of around 85 mg/l. The toxicity mechanisms of chromium, however, depend on its oxidation states and the pH of the system (Basu et al., 1997; Kotás and Stasicka, 2000). Chromium (III) could readily be precipitated from solution between pH 6–10, thus making it less available to biological systems. Due to high pH values of the sludge, it was presumed that the applied chromium (III) was precipitated out and the concentration of this metal in the activated sludge solution resulted as being very low and not significant as compared to those applied initially. Therefore, it was not available to the nitrifying population in the reactor. AUR experiments in the presence of chromium (III) revealed that this metal was not causing process inhibition during the pilot plant operations. It was demonstrated that the information from AUR could be used to identify possible inhibitory effects from wastewaters (Kristensen et al., 1992; Madoni et al., 1999). The information from AUR and NUR can thus be used as key parameters to determine the effectiveness of treatment processes and the biomass required to achieve desired effluent quality.
5.4 Enhancing biological nitrogen removal efficiency of the pilot plant by using the active Brachymonas denitrificans (IV)

The identification and characterization of the denitrifying bacteria responsible for nitrogen removal from wastewater treatment plants is complicated by the fact that 16S rRNA sequence-based identification of microorganisms does generally not allow inference of its functional properties. Phylogenetically closely related microorganisms may possess different metabolic potentials while, on the other hand, several physiological traits like the ability to denitrify are found in many different phylogenetic lineages. Therefore, *in situ* denitrifying activity measurement has to be supplemented with rRNA-approaches to monitor the members of the most important physiological groups in WWTPs. The combination of FISH and microelectrodes (Schramm et al., 1996; Okabe et al., 1999; Schramm et al., 1999, 2000), the recently developed combination of FISH and microautoradiography (Lee et al., 1999; Daims et al., 2001) and FISH and NUR (Paper IV) allowed the identification of the organisms responsible for important transformations of biological nutrient removal from wastewaters.

The aim of this study was to enhance biological nitrogen removal by introducing the efficient *Brachymonas denitrificans* (CCUG 45880) and to monitor *in situ* its efficiency, tolerance and abundance in the system. In both lab-scale and pilot plant experiments, NUR and FISH were used to determine the *in situ* activity of the active denitrifying bacteria and their abundance in the system. This bacterium was identified as tolerant to tannery effluent streams with toxic substances such as chromium and sulphide in addition to its remarkable ability to denitrify (Leta et al., 2004).
5.4.1 Identification of the efficient denitrifying isolates by fluorescent in situ hybridisation

Group- and species-specific DNA probes (Table 9) were used to evaluate *B. denitrificans* strains prior to in situ monitoring of the species. The species-specific probe S^-a-OTU6-0178-a-A-18 was used for in situ monitoring of the survival and dynamics of *B. denitrificans* after bio-augmentation. All the examined strains were identified as *B. denitrificans* in the β-subdivision of the *Proteobacteria* (Table 10, paper II).

Table 9. Oligonucleotide probes used for characterization and in situ detection of 16S rRNA sequences of *Brachymonas denitrificans* strains in this study

<table>
<thead>
<tr>
<th>Probe</th>
<th>Probe sequence from 5' to 3'</th>
<th>target organism(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAM42a</td>
<td>GCTTTCCACATCGTTT</td>
<td>γ-Proteobacteria</td>
<td>Munz et al. 1992</td>
</tr>
<tr>
<td>BETA42a</td>
<td>GCTTTCCACATCGTTT</td>
<td>β-Proteobacteria</td>
<td>Munz et al. 1992</td>
</tr>
<tr>
<td>ALF1b</td>
<td>CGTTGCYTCTGAGCCAG</td>
<td>α-Proteobacteria</td>
<td>Munz et al. 1992</td>
</tr>
</tbody>
</table>

Table 10. Evaluation of *Brachymonas denitrificans* isolates with group- and species-specific 16S rRNA probes

<table>
<thead>
<tr>
<th>Strains</th>
<th>Group-specific</th>
<th>Species-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BET42a</td>
<td>GAM42a</td>
</tr>
<tr>
<td><em>B. denitrificans</em> JCM9216</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>B. denitrificans</em> (CCUG 45880)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>B. denitrificans</em> (CCUG 45881)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>B. denitrificans</em> (CCUG 45882)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>B. denitrificans</em> (CCUG 45883)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>B. denitrificans</em> (CCUG 45884)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>B. denitrificans</em> (CCUG 45885)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Comamonas denitrificans</em></td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Strain JCM 9216 was obtained from Japan culture collection and the six strains were isolated from tannery wastewater treatment plant in Ethiopia. *C. denitrificans* was used as negative control for species-specific probe.

FISH analysis was performed as described by Amann (1995). Aliquots of 1 ml overnight cultures of *B. denitrificans* strains and sludge samples taken from the pilot plant, respectively, were centrifuged for 5 min at 9000 × g and the pellets were washed in 1xPBS. The cells were fixed in 4% paraformaldehyde fixative for 1.5 hours at 4°C. Three µl of the fixed cell suspensions were immobilized by air-drying on 9mm-diameter wells gelatine-coated slides. The cells were dehydrated by successive passages through 50, 80, and 98% ethanol washes for 3 min each. The cells were hybridized with probes as described by Amann (1995) at 46°C for two hours in 7.8 µl of hybridization buffer (1M Tris-Cl pH 7.4, 0.5m EDTA, 5M NaCl and 10% SDS, formamide (25%) with 1.2µl probe solution in a sealed moisture chamber. This was followed by a stringent washing step for 20 min in 50 ml of pre-warmed washing
buffer (1M Tris-Cl pH 7.4, 0.5m EDTA, 5M NaCl and 10% SDS). Slides were mounted with the antifade solution Citifluor glycerol/PBS (CITI FLUOR, UK) and the hybridized samples were examined under an epifluorescence microscope (Olympus BX51, Japan) attached to a CCD digital camera. AnalySIS® DOCU software (CC12 DOCU, Germany) was used for acquisition of images of hybridized cells (Figure 24).

**Figure 24.** Schematic flow chart of fluorescent in situ hybridization

FISH with a species-specific probe for *B. denitrificans* demonstrated the presence of this bacterium in the activated sludge of the pilot plant before bio-augmentation. However, dense populations of *B. denitrificans* were developed in the anoxic sludge of the pilot plant four days after introduction (Figure 25 a). The cell densities were clearly much higher than those before the addition of the bacterium. The abundance of the stained cells was consistent after its introduction for the three experimental runs (Figure 25a, b, c). Since *B. denitrificans* was isolated from tannery effluent, it should have the ability to tolerate different kinds of stresses present in such an environment, e.g. high salinity, chromium and sulphide. The tannery effluent would also contain a considerable amount of utilisable organic carbon, nitrogen and other nutrients (paper III) that may support the growth of this species in abundance in the system as determined by in situ hybridization technique.

*B. denitrificans* species possess high denitrifying capability in tannery wastewater activated sludge systems laden with such toxic substances as chromium (III) and sulphide (paper II). The increases in the denitrification rates of the sludge were consistent with the increase in the *B. denitrificans* population as determined by FISH. The bacterial populations remained abundant even after addition of the enrichment culture of *B. denitrificans* species was stopped. The presence of this species in large quantities in the anoxic system of the pilot plant could be explained by their versatile metabolism. Based on these observations, this species could be an important member of the denitrifying population in this system. Satoh et al.
(2003) also observed that addition of enrichment cultures of nitrifying bacteria into a biofilm reactor facilitated development of dense nitrifying bacterial populations, which led to a rapid start-up of the process and enhancement of *in situ* nitrification activity.
5.4.2 Denitrifying activity measurements

The denitrifying activity of the sludge with and without the addition of *B. denitrificans* was monitored both in lab scale reactors and in pilot plant experiments as described earlier. Enrichment cultures of *B. denitrificans* were added to one of the reactors with respective cell densities of 2.5x10⁸, 1.5x10⁹, and 1.8x10⁹ c.f.u. ml⁻¹ for experiments I, II and III, respectively. The other reactor was operated without the addition of the bacteria and NO₃-N was added to both reactors at a concentration of 60 mg/l to be removed. NUR was determined as mg NO₃-N gVSS⁻¹h⁻¹.

The experiment was repeated *in situ* in the pilot plant. Before the addition of the active denitrifying population into the anoxic system of the pilot plant, NO₃-N was added at a concentration of 90 mg/l to be removed in order to know the denitrifying capacity of the sludge and compare this with that of the sludge bio-augmented with the bacterium. After two weeks of the NUR experiments with the sludge only, the pilot plant was augmented with enrichment culture of *B. denitrificans* with respective cell densities of 6.7x10⁸, 4.8x10⁹, 5.8x10⁹ c.f.u. ml⁻¹. The same concentration of NO₃-N was also added to be removed and NUR was monitored as mg NO₃-N gVSS⁻¹day⁻¹ and the dynamics of the introduced bacterium was monitored by species-specific 16S rRNA-targeted probe.

Both lab scale and pilot plant NUR measurements showed a higher denitrifying activity as compared with the NUR results before bio-augmentation with *B. denitrificans*. The denitrifying activity of the sludge augmented with the active bacterium for all the lab scale experiments was slightly higher (2.9±0.1–4.5±0.2 mg NO₃-N gVSS⁻¹h⁻¹) as compared with the sludge without the bacteria (2.5±0.1–4.2±0.2 mg NO₃-N gVSS⁻¹h⁻¹) (Figure 26a). The mean values for augmented and non-augmented sludges were 3.7±0.6 and 3.5±0.7 mg NO₃-N gVSS⁻¹h⁻¹, respectively. Similarly, the *in situ* denitrifying activity of the sludge after the
Introduction of active denitrifying population was higher (10.8±0.5–14.2±0.7 mg NO$_3$-N gVSS$^{-1}$day$^{-1}$) as compared with the activated sludge before bio-augmentation (8.9±0.4–12.9±0.6 mg NO$_3$-N gVSS$^{-1}$day$^{-1}$) (Figure 25b). The mean NUR values with and without bio-augmentation were 12.0±1.4 and 10.6±1.4 mg NO$_3$-N gVSS$^{-1}$day$^{-1}$, respectively. Both experiments indicated that bio-augmentation using this denitrifying bacterium could enhance the removal of nitrogen from tannery effluents characterized by high levels of toxic substances.

![Figure 26. Denitrification rate of the activated sludge with and without Brachymonas denitrificans in lab scale (a) and pilot plant (b) experiments. Data are means of three measurements and error bars represent their standard deviations.](image)

It was noted that the nitrogen removal capacity between the augmented and non-augmented sludges was not significant (Figure 26a & b). These small differences perhaps can be attributed to the fact that the efficient bacterium, B. denitrificans, is already present in the pilot plant system before introduction. The addition of NO$_3$-N can also stimulate the indigenous denitrifying organisms including B. denitrificans found in the system. Increasing cell densities of the inoculum might also have a greater effect on the observed N removal rate in both conditions. The population size and activity of the degradative bacteria plays an important role in the reduction of specific pollutants in wastewater treatment (Goldstein et al., 1985). It should also be noted that the system might have encouraged the growth of high consortia of denitrifiers to attain high denitrification efficiency (paper III). Hence bio-augmentation was likely not to have had a greater effect than observed in the present study.

However, because acclimatized sludge was used in this study, further research on the effect of bio-augmentation of non-acclimatized sludge for start-up of processes and during process failures should be investigated for wider acceptance of bio-augmentation of this species in wastewaters laden with toxic substances.

Bio-augmentation with desired catabolic traits can be effective for biodegradation of pollutants (Rittmann and White, 1994). This operational tool does, however, frequently fail (Bouchez et al., 2000). Such failure is typically by addition of the “wrong” microorganisms that cannot compete successfully with autochthonous bacteria in the plant.
and are thus eliminated or washed out. Reports have showed that the survival, in situ activity and maintenance of introduced microorganisms are influenced by such factors as selection of suitable strains, effective attachment, development of active population of introduced bacteria and effluent composition (McClure et al., 1991; Watanabe et al., 1998; Bouchez et al., 2000). The importance of selecting bacterial inoculants with appropriate physiological characteristics for survival and in situ activity in target ecosystems was demonstrated in this study (paper IV).

It is interesting to note that a denitrification potential of up to 12.9±1.8 mg NO\textsubscript{3}-N gVSS\textsuperscript{-1}day\textsuperscript{-1} was measured in run III in the activated sludge of the pilot process plant before bioaugmentation. When augmented with the active denitrifying population, the denitrification rate was increased to 14.2±1.6 mg NO\textsubscript{3}-N gVSS\textsuperscript{-1}day\textsuperscript{-1} (Figure 26b) showing that bioaugmentation strategy can enhance the removal of target pollutants from tannery effluents. The ability to grow in systems of a toxic nature might lead to the successful establishment and activity of the \textit{B. denitrificans} population in the system in addition to their peculiar performances in such wastewater environments (paper II). Bio-augmentation using appropriate bacteria has also been shown to enhance biodegradation rates for persistent wastewater constituents from a mixed landfill leachate and chemical industry wastewater (Ying et al., 1986).

In this study, a clear correlation was observed between the in situ denitrifying activity measured by NUR, population dynamics of the introduced \textit{B. denitrificans} monitored by FISH and the pilot plant system performance, suggesting that the strategy of introducing this species for enhancing process performance has potential applications.

5.5 Potential of the pilot process plant for biological sulphide removal (paper V)

Sulphur pollution can be controlled by microbial oxidation of sulphide to elemental sulphur. Consortia of chemolithotrophic microorganisms in anoxic environments accomplish this transformation. Two major types of nitrate-reducing bacteria (NRB) can be stimulated in an anoxic nitrate rich environment: one is the chemoorganotrophic (heterotrophic) NRB that use organic compounds as electron donors. The second type is the chemolithotrophic nitrate-reducing, sulphide-oxidizing bacteria (NR-SOB) (Eckford and Fedorak, 2002). Numerous studies have been conducted to remove sulphide from different sulphide waste environments by adding nitrate (Jenneman et al., 1986; Londry and Suflita, 1999; Davidova et al., 2001). The predenitrification followed by nitrification process of the pilot plant facilitated this biogenic sulphur transformation in this study. The sulphide removal study was performed as part of the overall performance assessment of the pilot process plant for the treatment of tannery effluent (paper V).

The ability to grow chemolithotrophically on reduced sulphur compounds is a property of a diverse group of sulphur-oxidizing bacteria in the genera of \textit{Thiobacillus}, \textit{Beggiaota}, \textit{Thiobrix}, \textit{Thioploca}, \textit{Thermosipiribacter}, \textit{Thiosphaera}, \textit{Thermobrix} and \textit{Thiovalum} (Madigan et al., 2002). Most sulphur-oxidizing bacteria can grow well around neutral pH. Some sulphur-oxidizing bacteria such as \textit{Thiobacillus denitrificans}, \textit{Thioploca}, \textit{Thiomicronspira denitrificans}, \textit{Thermobrix} and \textit{Thiosphaera} can use nitrate as an electron acceptor in anoxic conditions and thus may be enriched in such environments. Studies on the ecology of these organisms have shown that they carry out the anoxic oxidation of sulphide coupled to the reduction of nitrate presumably to N\textsubscript{2} (denitrification) (Cloete and Muyima, 1997; Gevertz et al., 2000; Madigan et al., 2002). Under oxygen limited conditions, that is, dissolved oxygen concentrations below 0.1 mg/l, elemental sulphur is the major end product of the sulphide oxidation, while sulphate is formed under sulphide-limiting conditions (Janssen et al., 1995).
5.5.1 Biological sulphur transformations in the pilot process plant

The pilot process plant showed remarkable sulphide removal efficiency in spite of high influent perturbations, indicating that biological treatment of tannery wastewater containing a high load of sulphide is possible. The removal efficiencies of the pilot plant for sulphide and chromium (III) ranged from 95–99% and 91–96%, whereas it was 91–95% and 94–96% for COD and total N, respectively. Interestingly, a steady removal efficiency of sulphide was observed throughout the study period. It was also seen that sulphide removal efficiency was consistent with nitrogen and COD removal efficiencies of the system (Figure 27). The pilot plant was performing at a COD and sulphide loading rates of 0.8–1.8 kg m⁻³ day⁻¹ and 8.1–37.75 gm m⁻³ day⁻¹, respectively, during the study period.

![Graph of sulphide, Total N, COD and chromium (III) removal efficiencies of the pilot plant](image)

**Figure 27.** Sulphide, Total N, COD and chromium (III) removal efficiencies of the pilot plant

It is interesting to note that during this biogenic sulphur transformations in the pilot process plant, the sulphate concentrations increased from the initial influent concentrations especially in the oxic and effluent streams while sulphide decreased significantly in the anoxic system of the pilot plant, consistent with sulphide oxidation as observed in Figure 28a. The result showed that sulphide removal was mainly accomplished in the anoxic system of the pilot plant. It was also shown that sulphide formation from the reduction of sulphate was not significant indicating that the activities of sulphur-reducing bacteria in the system were inhibited. It is interesting to note that the oxic and effluent streams of the pilot plant showed a slight increase in the sulphate concentrations, which might have resulted from sulphide oxidation in the anoxic and aerated compartments (Figure 28b).

The high chromium removal efficiencies observed in the system were due to a long retention time and favourable reactor pH values, which was close to the optimum pH for lime precipitation of trivalent chromium (Tadesse, 2003). In the anoxic system of the pilot process plant, where the pH was maintained between 8–9, further settling of chromium as chromium (III) hydroxide could also take place.
5.5.2 Nitrate-reducing, sulphur-oxidizing bacteria

Nitrate-reducing, sulphur-oxidizing bacteria (NR-SOB) were enumerated by colony-forming units (c.f.u.) technique. The NR-SOB were estimated to vary from 4.8 x 10^4 to 7.8 x 10^5 c.f.u. ml^-1 indicating that the sulphur-oxidizing bacteria present in the system were those that can use reduced inorganic sulphur compounds as electron donors and nitrate as an electron acceptor. An increase in the population size of the NR-SOB in the activated sludge of the pilot plant was correlated with a significant removal of sulphide from the effluent throughout the study period (Figure 29). Sulphide concentrations in the influent wastewater ranged from 67.5–377.5 mg/l and the corresponding sulphide effluent concentrations ranged from 0.07–0.8 mg/l for the months of August to November (paper V). Effluent sulphide concentrations in June and July were 20.5 mg/l and 1.1mg/l, respectively, and the corresponding NR-SOB population were also low with removal efficiencies of 95% and 98%. Subsequently, the sulphide removal efficiencies of the system remained 99% with relatively increasing NR-SOB population densities in the system.

The presence of NR-SOB in the activated sludge of the system not only removed sulphide, but also suppressed sulphide formation by sulphate-reducing bacteria (SRB) in the system. The anoxic condition in the presence of NO_3-N stimulates the nitrate-reducing; sulphur-oxidizing bacteria to remove sulphide as well as stimulating heterotrophic denitrifiers that can out-compete SRB for energy sources. No sulphate reduction by the system was obtained throughout the study period (Figure 28b). The sulphate concentrations increased in the effluent compared with initial influent sulphate concentrations while the sulphide was steadily removed from the system (Figure 28a). These observations indicated that the activity of NR-SOB suppressed sulphate reduction and contributed to the removal of sulphide originally present in the influent wastewater. Studies have also shown that the presence of nitrate reducing bacteria and the use of nitrate to control sulphate reduction in sulphur-rich wastewaters (Telang et al., 1997; Jenneman et al., 1999; Gevertz et al., 2000).
From these observations, NR-SOB could play a key important function in sulphide and nitrogen laden tannery effluents. The presence of chemolithotrophic NR-SOB in the activated sludge of the pilot plant treating tannery effluent provides opportunities for the application of biologically mediated simultaneous removal of sulphide and nitrogen from tannery effluents. Tannery wastewater is rich in reduced sulphur compounds and nitrogen and is thus an ideal environment for such denitrifying population in the system. Reports demonstrated that addition of nitrate to sulphide laden-wastes have been used to remove sulphides from these wastes (Jenneman et al., 1986; 1999; Eckford & Fedorak, 2002). It is, therefore, necessary to conduct further specific, molecular-based studies in order to specifically assess which NR-SOB populations proliferated in such environment and play a key role in controlling sulphide concentrations in tannery WWT system.

The typical performance of the pilot process plant may allow the proposal of new integrated nitrogen and sulphide removal systems. Many reports (Bentzen et al., 1995; Gevertz et al., 2000) indicate that the use of nitrate to control H2S odours in sewers and other wastewater environments could have commercial applications. In this study, it has been shown that a predenitrification–nitrification process can be used as an alternative method for the mitigation and control of sulphides in tannery effluents. In addition to enriching heterotrophic denitrifiers for nitrogen removal, the anoxic predenitrification process stimulated the activity of indigenous NR-SOB to simultaneously remove sulphide from the system.
6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

It has been shown that the leather industry generates a strong wastewater with high COD, BOD₅, total N, sulphide and ammonia concentrations. Furthermore, trivalent chromium is a major pollutant of concern. Nitrogen in its various forms can have toxicity effects on aquatic life forms, contributes to eutrophication of the receiving waters and also becomes a concern if the receiving stream is to be used downstream as a source of drinking water.

The predenitrification-nitrification process developed and used in this study was found to be efficient for the treatment of tannery effluents containing high concentrations of degradable organic carbon, nitrogen and ammonia. This process enables optimal use of the incoming tannery influent as carbon source for denitrification besides protecting the sensitive nitrifying organisms from toxic shocks and helps to achieve high degrees of nitrification. Despite high chromium and sulphide contents in the influent, high removal efficiencies were obtained for total N and COD ranging from 82-98% and 95-98 %, respectively. Up to 95% ammonium N removal efficiency was also obtained in the pilot plant although the nitrification performance of the system was low at the start-up.

Moreover, a steady removal efficiency of sulphide was observed to be consistent with the abundance of NR-SOB in the system. In this study, it has been shown that the predenitrification-nitrification can be used as an alternative method for the mitigation and control of sulphide pollution. In addition to enriching heterotrophic denitrifiers for nitrogen removal, the anoxic predenitrification process stimulated the activity of indigenous NR-SOB to simultaneously remove sulphide from the system. The typical performance of the pilot plant may allow the proposal of new integrated nitrogen and sulphide pollution control strategy. The six *B. denitrificans* isolates characterized in this study are also of great value because they are tolerant to tannery effluent streams with toxic substances in addition to their remarkable denitrifying properties. Hence further full-scale research should be carried out for wider application of this process as well as *B. denitrificans* in wastewaters laden with toxic substances.

A pilot bioprocess plant developed and operated for nutrient removal from tannery effluents is a good example of a model environmental-scale bioreactor system. By making use of novel microorganisms, application of this process to industry can turn problems associated with tannery effluents into opportunities. Further research endeavors should, therefore, be supported by a strong emphasis on technology transfer from laboratory studies to full-scale industrial applications based on the experiences and findings developed so far from this study. In addition to transfer and application of bioprocess technologies to industry to improve and manage the environment in a sustainable manner locally, the future perspective is also to create a strong research and training linkages through exchange of information and expertise in the region and at international levels.
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