Nitrification and oxidation of carbon sources and nitrogen removal in ammonia rich effluents

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Dissertação para a obtenção do Grau de Mestre em Engenharia Biológica

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAS</td>
<td>Aerobic Activated Sludge</td>
</tr>
<tr>
<td>ABIL</td>
<td>Ammonia Binding Inoculum Liquid</td>
</tr>
<tr>
<td>AnAOB</td>
<td>Anoxic Ammonia Oxidizing Bacteria</td>
</tr>
<tr>
<td>AOB</td>
<td>Aerobic Ammonia Oxidizing Bacteria</td>
</tr>
<tr>
<td>CANON</td>
<td>Completely Autotrophic Nitrogen Removal over Nitrite</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>CS</td>
<td>Carbon Source</td>
</tr>
<tr>
<td>DE</td>
<td>Dranco Extract</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved Oxygen</td>
</tr>
<tr>
<td>HA</td>
<td>Humic Acids</td>
</tr>
<tr>
<td>ISA</td>
<td>International Searching Authority</td>
</tr>
<tr>
<td>NOB</td>
<td>Nitrifying Bacteria</td>
</tr>
<tr>
<td>NI</td>
<td>Nitrifying Inoculum</td>
</tr>
<tr>
<td>OLAND</td>
<td>Oxygen-Limited Autotrophic Nitrification/Denitrification</td>
</tr>
<tr>
<td>OUR</td>
<td>Oxygen Uptake Rate</td>
</tr>
<tr>
<td>RBC</td>
<td>Rotating Biological Contactor</td>
</tr>
<tr>
<td>SHARON</td>
<td>Single Reactor System for High Ammonia Removal Over Nitrite</td>
</tr>
<tr>
<td>TAN</td>
<td>Total Ammonia Nitrogen</td>
</tr>
<tr>
<td>TSS</td>
<td>Total Suspended Solids</td>
</tr>
<tr>
<td>VSS</td>
<td>Volatile Suspended Solids</td>
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Thesis outline

This thesis includes three independent works about nitrification and denitrification processes as well as the ability of chemical oxygen demand (COD) removal by certain types of enriched inocula.

The first work is about several types of nitrifying inocula (NI) that are currently used in aquaculture and in domestic and industrial aquaria. The accumulation of ammonia appears to be toxic to all the vertebrates and in the case of fish is often a problem limiting the aquaculture production or domestic aquaria maintenance. Several nitrifying inocula were tested in order to determine their specific activity concerning total ammonium nitrogen (TAN) and nitrite removal. A critical review of the patents that have been granted on this type of product is also presented. Concerning one specific NI, the ammonia-binding liquid inoculum (ABIL), several tests were performed in order to evaluate its heterotrophic activity. The activity of oxidation of several carbon sources (CS) was determined and compared with the activity of aerobic activated sludge from three different origins. Since this inoculum is autotropically fed, a simple test based on the oxygen uptake rate (OUR), was developed in order to allow fast distinction between ABIL and regular activated sludge.

Concerning the second part of this thesis, the start-up of an oxygen-limited autotrophic nitrification denitrification (OLAND) rotating biological contactor (RBC) reactor was followed-up. In order to remove nitrogen from ammonium rich effluents, several solutions have been developed as alternatives to the most common nitrification-denitrification systems. These “non-conventional” nitrogen removal systems present some advantages over the typical systems, namely energy and extra COD savings. The OLAND system is based in a biofilm constituted by two major groups of bacteria. These two types of organisms are able to perform in a single step, the oxidation of ammonia into nitrite and the subsequent conversion of a stoichiometric amount of ammonia and nitrite into dinitrogen gas. In this work, the influence of several operational conditions on the start-up period of the reactor was evaluated for about 200 days. The influence of adding different supplements to the synthetic influent of the reactor was also studied.

The last part of this thesis is about the enrichment of an inoculum that could be hypothetically mixed with a de-icer in order to allow faster degradability of the same. The de-icing techniques are commonly used in order to avoid ice accumulation in vulnerable environments such as roads and airports. The COD overload that this type of application can generate in the environment, stimulated the development of a system that could allow faster degradation of the de-icer in the place where usually it is applied. After growing a certain amount of activated sludge in a batch reactor, several activity tests were performed in order to evaluate the degradation potential of the inoculum. The possibility of
storing the product by freezing it at -20ºC was assessed. Activity tests were also performed in order to evaluate the ability of this inoculum to retain its activity after a freezing step and being mixed afterwards with a commercial de-icer.
1 Literature review

1.1 Aquaculture

When compared with other animal food producing industries, aquaculture is growing faster. The contribution of aquaculture to the global fisheries has been increasing from 3.9% in 1970 to 34% in 2005.

Given a total worldwide production of 141 million tonnes, aquaculture contributed with 48 million tonnes by weight and US$ 78.4 billion by value. This increasing growth rate (> 10% per year for most species) contrasts with the stagnant contribution from wild fisheries during the last decade. At the same time, production from aquaculture has greatly outpaced population growth, with its per capita supply increasing from 0.7 kg in 1970 to 7.1 kg in 2004 at an average annual growth rate of 7.1 percent (FAO, 2005).

The ornamental fish sector is yet a small but important component of the aquaculture business. The scope of this sector is often unappreciated. The value of the entire ornamental fish industry including non-exported product, wages, retail sales and associated materials has been estimated at US$ 15 billion or approximately 20% of the total value of the aquaculture business (FAO, 2000). A survey from 2004 indicates that in the United States 15 million households are used to own a certain amount of saltwater or freshwater fish. Thus the total number of fish individuals was estimated in 152 million (2004, APPMA).

Currently, approximately 90% of freshwater fish traded in the hobbyist industry are captively cultured (Tlustly, 2002).

Even considering a scenario of low growth rates in the global fisheries sector, the contribution of aquaculture to this business will keep the same increasing trend. Indeed, for the next years, most of the increase in fish production is expected to come from aquaculture.
Intensification of aquaculture will increase the biomass load per m$^3$ in order to increase the fish yield. Ammonia concentration appears to be the most important factor limiting the amount of fish per water volume given its toxicity for fish. Recirculation systems with different types of treatment allow the increase of biomass for a certain volume of water (Meade, 1985). Most of the fish excrete ammonia not only by the gills but also through the skin and faeces. At the same time, food wastage contributes directly for the increase of ammonia. In general, a value between 52 and 92% of the total N added as food is thought to pollute lately the environment (considering both wastage and the amount consumed by fish) (Handy & Poxton, 1993). Thus, the use of recirculation devices accompanied by effective treatment strategies is a critical improvement to apply in efficient aquaculture systems.

### 1.2 Toxicity of Ammonia

Elemental nitrogen can assume several different forms. The proteinaceous nitrogen is present in the most reduced form (-3) like in the ammonia molecule and ammonium ion. The oxidation state is +3 for nitrite and + 5 for nitrate (the most oxidized forms of nitrogen). Since its importance in terms of water quality and also because these three inorganic species contain only one atom of nitrogen, the presence of the molecules is always expressed in terms of the nitrogen weight (eg.: N-NH$_3$ or N-NH$_4^+$).

In water, gaseous ammonia (NH$_3$) is in equilibrium with the ammonium ion (NH$_4^+$) according to:

$$ NH_4^+ + H_2O \rightleftharpoons NH_3 + H_3O^+ $$

$$ \text{pK}_a = 9.24 \ (T=25^\circ C) \quad \text{(Hargreaves, 1998)} \quad (1) $$

This equilibrium is highly dependent on the pH and temperature. High pH and high temperature shift the equilibrium towards ammonia. The sum of N-NH$_3$ and N-NH$_4^+$ is called total ammonium nitrogen (TAN) (Hagopian & Riley, 1998).

According to Anthonisen, the relative amount of TAN in solution, present as ammonia can be calculated as:

$$ \text{NH}_3 = \frac{1.214 \times \text{TAN} \times 10^{\text{pH}}}{e^{6346 \over 273+T} + 10^{\text{pH}}} \quad \text{(Anthonisen et al, 1976)} \quad (2) $$

All the values are given in mg/L with temperature $T$ in ºC between 0 and 45ºC.

Ammonia is toxic to all the vertebrates by inhalation, skin contact or ingestion and its effects can vary from mild eye irritation (50 ppm) to eyes injuries (700 ppm) or dead (2500 ppm) (Issley, 2007).

When exposed to un-ionized ammonia, fish show nervous system disturbances, increased gill ventilation, loss of equilibrium, convulsions and high mortality (Foss et al, 2003).
Exposure to low concentrations of ammonia can also generate a decrease in the growth rate. Table 1.1 presents a summary on LC\textsubscript{50} values for some aquatic organisms.

**Table 1.1.** Summary on LC\textsubscript{50} values for some cultured aquatic organisms, given a period of time of 96 h (Person-Le Ruyet et al, 1995).

<table>
<thead>
<tr>
<th>Organism</th>
<th>LC\textsubscript{50} (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TAN</td>
</tr>
<tr>
<td>Dicentrarchus labrax</td>
<td>40</td>
</tr>
<tr>
<td>Scophthalmus maximus</td>
<td>59</td>
</tr>
<tr>
<td>Sparus aurata</td>
<td>57</td>
</tr>
</tbody>
</table>

1.3 **The nitrogen cycle: autotrophic nitrification**

The main natural sources of environmental ammonia are nitrogen fixation and ammonification or mineralization. Nitrogen fixation (Figure 1.1, 2) is the process by which atmospheric nitrogen is converted to ammonia. Subsequently by assimilation ammonia will be converted in other forms of organic nitrogen (Figure 1.1, 3). This organic nitrogen will be transformed in ammonia and a carbon component by mineralization (ammonification) (Figure 1.1, 4) (Postgate, 1998).

![Figure 1.1 Nitrogen cycling and turnover](image)

Conversion of ammonia to nitrate occurs by a process called nitrification. Autotrophic nitrification is a two step process by which ammonia is biologically oxidized to nitrite (nitritation) and then to nitrate (nitratation) (Kowalchuk & Stephen, 2001).

The global equation for the aerobic nitrification process can be expressed as:

$$NH_3 + 2O_2 \rightleftharpoons NO_3^- + H_2O$$
Despite being a key process in nitrogen cycle, increasing nitrite and nitrate availability has considerable impact on groundwater pollution as a result of leaching (Leininger, 2006). From an anthropocentric point of view, nitrification can be considered both detrimental and beneficial. The conversion of ammonia and urea fertilizers to nitrate contributes to fertilizer loss from agricultural soils by producing compounds that can be easily washed-out (Purkhold, 2000). The two steps of biological nitrification are catalyzed by two phylogenetically unrelated groups of chemolitho-autotrophic bacteria: the ammonia-oxidizing bacteria (AOB) and the nitrite-oxidizing bacteria (NOB) (Figure 1.2).

![Figure 1.2 Distance tree for the proteobacteria, including some nitrifying isolates. The scale bar (10%) corresponds to 0.1 estimated fixed mutation per sequence position (Teske et al, 1994).](image)

### 1.4 Ammonia-oxidizing bacteria (AOB)

Chemolitho-autotrophic nitrifying bacteria were used to be comprised in one family called Nitrobacteraceae.

The first four genera of AOB’s, namely *Nitrosomonas*, *Nitrosospira*, *Nitrosocystis* and *Nitrosogloboea* were described by Winogradsky in 1892.

The two genera *Nitrosogloboea* and *Nitrosocystis* contained bacteria used to grow in aggregates. This characteristic was dependent on the culture conditions, therefore was not considered a suitable taxonomic criterion. AOB were then grouped in the two genera *Nitrosomonas* (containing rod-shaped organisms) and *Nitrosospira* (containing spiral-shaped organisms).

Later, several species were assigned to the *Nitrosospira* and *Nitrosomonas* genera.

At the same time, new species that had been isolated were assigned to the genus *Nitrosococcus* introduced by Migula et al in 1900 (Watson, 1971; Vanparys, 2006). *Nitrosovibrio tenuis* was also described as a species belonging to a distinct genus by Harms et al (1976).
Later, typical taxonomic approaches based on morphological aspects were substituted by using 16S rRNA oligonucleotides and 16S rRNA gene sequencing analysis (Vanparys, 2006). Currently, concerning AOB and according to 16S rRNA gene sequence data, all known aerobic AOB isolates were restricted to two monophyletic groups within the β and γ subclasses of the proteobacteria (Head et al., 1993). The γ-subclass contains only the genus *Nitrosococcus* with two organisms described so far (*N. oceani* and *N. halophilus*). The β-subclass of the proteobacteria comprises organisms from the genera *Nitrosomonas* and *Nitrosospira* (that includes also *Nitrosovibrio tenuis* and *Nitrosolobus*) (Koops & Pomerening-Röser, 2001). A list of all the 17 currently valid species is shown in Table 1.2. A dendogram based on 16S rDNA sequences as well as some ecophysiological parameters for AOB is shown in Figure 1.3.

**Table 1.2.** List of the 17 currently valid species within the AOB (Vanparys, 2006).

<table>
<thead>
<tr>
<th>Nitrosoberoccus</th>
<th>Species designation</th>
<th>Nitrosomonas</th>
<th>Species designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. halophilus</td>
<td>N. aestuani</td>
<td>N. communis</td>
<td>N. cryotolerans</td>
</tr>
<tr>
<td>N. mobilis</td>
<td>N. europaea</td>
<td>N. eutropha</td>
<td>N. halophila</td>
</tr>
<tr>
<td>N. nitrosus</td>
<td>N. briensis</td>
<td>N. marina</td>
<td>N. nitroa</td>
</tr>
<tr>
<td>N. oceani</td>
<td>N. multiformis</td>
<td>N. oligotropha</td>
<td>N. ureae</td>
</tr>
</tbody>
</table>

**Figure 1.3.** Dendogram based on 16S rDNA, showing the phylogenetic interrelationship among the cultured AOB. The tree was constructed by using the neighbour-joining method. Included is information on ecophysiological parameters and preferred habitats (Koops & Pomerening-Röser, 2001).
Although the AOB are obligate aerobic autotrophs, some species may be tolerant to low oxygen or anoxic environments. They use ammonia as the only source of energy and carbon dioxide as carbon source.

Conversion of ammonia to nitrite by AOB consists in two steps. Hydroxylamine is obtained as intermediary compound and further converted to nitrite. Conversion of hydroxylamine to nitrite generates energy.

\[
\begin{align*}
NH_3 + O_2 + 2H^+ + 2e^- \xrightarrow{AMO} NH_2OH + H_2O & \quad \Delta G = +17 \text{ kJ/mol} \\
NH_2OH + H_2O \xrightarrow{HAO} NO^-_2 + 5H^+ + 4e^- & \quad \Delta G = -292 \text{ kJ/mol} \\
0.5O_2 + 2H^+ + 2e^- \xrightarrow{\text{terminal-oxidase}} H_2O & \\
NH_3 + 1.5O_2 \rightarrow NO^-_2 + H^+ + H_2O & \quad \Delta G = -275 \text{ kJ/mol}
\end{align*}
\]

First reaction is catalysed by a membrane-bound, multisubunit enzyme called ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) catalyzes the second.

Two of the electrons produced in the second reaction are used to compensate the input of electrons in the first reaction. The two remaining electrons are passed via an electron transport chain to the terminal oxidase, generating a proton motive force.

Although all the species of AOB use ammonia as sole energy source, their substrate affinity (\(K_s\) value for the ammonia-oxidizing system) differs significantly among the species reflecting well phylogenetical identities.

Competition between the different types of AOB is mainly dependent on their relative rate of substrate utilization. Therefore, two distinct strategies can be identified in terms of saturation kinetics: \(R\)- and \(K\)-strategy. \(R\)-strategists have high maximum specific growth and substrate utilization rates at high substrate concentrations. \(K\)-strategists have substrate affinity at low substrate concentrations.

Given a \(K_m\) value for Nitrosomonas, 1-2 orders of magnitude higher than for Nitrosospira, the later is classified as a \(K\)-strategist (Figure 1.3).

Despite their ability to grow in organic substrates, some species can, through urea hydrolysis, obtain a source of ammonia (Figure 1.3). In general AOB are found in aerobic environments where ammonia is available through ammonification processes thanks to anthropogenic sources like fertilizers or waste (Kowalchuk & Stephen, 2001).

According to Kowalchuk & Stephen (2001) both species of Nitrosococcus and several Nitrosomonas species are obligate halophilic. Despite this consideration, \(N.\) marina-like strains were detected in freshwater aquaria biofilters. \(N.\) marina related strains were also found to be
the most efficient in decreasing the start-up time of freshwater aquaria biofilters (Burrell et al, 2001).

In freshwater aquaria biofilters, initially dominated by *N. marina* related strains it was possible to notice after several weeks of operation the maintenance of the same initial *N. marina* strains surrounded by a closely related diversified population (Grommen, 2005).

In general, a medium containing low substrate concentrations (up to 8 mg N-TAN/L) can give large Most-Probable-Number (MPN) counts of AOB than media containing high TAN concentrations. Natural environments, such as soil or water, contain in general concentrations ranging from 1 to 10 mg N-TAN/L. Concentration of free ammonia (NH$_3$) rather than the TAN concentration inhibits AOB. As mentioned above, the equilibrium between the two forms is strongly dependent on the pH. Therefore, toxicity of TAN will also depend on the pH (Princic et al, 1998).

The effect of pH on the affinity constant for ammonia was found to decrease markedly with increasing pH. Therefore, ammonia is generally accepted as the substrate of the reaction. (Suzuki et al, 1974).

Further in this text, AOB stands always for aerobic ammonia oxidizing bacteria. In order to refer to anoxic AOB, the acronym AnAOB was used. The term *anoxic* was used, instead of *anaerobic* since generally in wastewater treatment, the absence of oxygen is indicated as *anoxic*. The term *anaerobic* is used to indicate the absence of any electron acceptor (nitrate, sulphate or oxygen).

**1.5 Nitrite-oxidizing bacteria (NOB)**

The first genera of NOB were isolated a long time ago by Winogradsky (1892). The original strains as described by Winogradsky were not preserved in laboratory culture. Strains belonging to the genera *Nitrocystis* were reserved for bacteria used to grow in aggregates. Later was possible to observe that a large amount of nitrifiers could also adopt this strategy according to the culture conditions. Therefore this characteristic was not considered a reliable taxonomic criterion and *Nitrocystis* was not considered anymore a valid name (Watson & Mandel, 1971).

Strains possessing rod to pear-shaped cells, having a polar cap, cytomembranes and reproducing by budding were assigned to the genus *Nitrobacter* having *N. winogradsky* as their major representative. Some authors found that the high serological diversity among isolates from the genus *Nitrobacter* was greater than that covered by the four species of the genus (Vanparys, 2006).

Till now, four phylogenetically distinct groups of NOB have been described. The major group is represented by the *Nitrobacter* genus and belongs to the α-subclass of the proteobacteria. The two marine species *Nitrooccus mobilis* and *Nitrospina gracilis* were assigned to the γ and δ subclass of proteobacteria, respectively. The two species of the genus *Nitrospira* were assigned to a distinct phylum close to the δ subclass of proteobacteria (Koops & Pommerening-Röser,
2001). A list of the 8 current valid species is shown in Table 1.3. Recently, provisionally classified Candidatus *N. efluvii* was obtained by enrichment and was found to be a representative of a distinct sublineage inside the genus *Nitrospira* (Spieck, 2006). A summary on the phylogenetic relationships among NOB genus as well as some ecophysiological parameters is shown in Figure 1.4.

**Table 1.3** List of the 8 currently valid species within the NOB (Vanparys, 2006).

<table>
<thead>
<tr>
<th>Species Designation</th>
<th>Species Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrobacter</td>
<td>Nitrospina</td>
</tr>
<tr>
<td>N. alkalicus</td>
<td>N. gracilis</td>
</tr>
<tr>
<td>N. hamburgensis</td>
<td></td>
</tr>
<tr>
<td>N. vulgaris</td>
<td></td>
</tr>
<tr>
<td>N. winogradsky</td>
<td>N. marina</td>
</tr>
<tr>
<td>Nitrococcus</td>
<td>Nitrospira</td>
</tr>
<tr>
<td>N. mobilis</td>
<td>N. moscoviensis</td>
</tr>
<tr>
<td></td>
<td>candidatus N. efluvii</td>
</tr>
</tbody>
</table>

**Figure 1.4** Dendogram based on 16S rDNA showing the phylogenetic interrelationship among the cultured NOB. The tree was constructed by using the neighbour-joining method. Included is information on ecophysiological parameters and preferred habitats (Koops & Pommerning-Röser, 2001).

The NOB are inhibited in case of high concentrations of free ammonia (Gieseke et al, 2003) and in low DO environments (Hanaki et al, 1990). These nitrifiers obtain energy by oxidizing nitrite to nitrate using a membrane-bound nitrite oxidoreductase (NOR). Despite the possibility of using nitrite as major energy source, all the members from the *Nitrobacter* genus are able to use organic sources such as pyruvate (Bock and Koops, 1992).

The nitrite oxidation reaction provides two electrons that are passed via an electron transport chain to the terminal oxidase, generating a proton motive force:
\[ NO_2^- + H_2O \xrightarrow{\text{NOR}} NO_3^- + 2H^+ + 2e^- \quad \Delta G = -75 \text{ kJ/mol} \quad (7) \]

\[ 0.5O_2 + 2H^+ + 2e^- \xrightarrow{\text{terminal-oxidase}} H_2O \quad (8) \]

\[ NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad \Delta_G = -75 \text{ kJ/mol} \quad (9) \]

1.6 Ecophysiology of AOB and NOB

In Table 1.4, some of the most important physiological parameters for the two groups of nitrifiers are shown. Both AOB and NOB present low growth rates with generation times up to 3 days. Conditions that can be found in aquaria and aquaculture systems are close to the optimal. However and given the high toxicity of ammonia and nitrite to aquatic organisms, this bacteria often experience substrate concentrations that can be below the optimal value of their affinity constant (Grommen, 2005).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AOB</th>
<th>NOB</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generation time (d)</td>
<td>0.5-1.7</td>
<td>0.5-2.5</td>
<td>Belser &amp; Smith (1980); Focht &amp; Verstraete (1977)</td>
</tr>
<tr>
<td>Specific Growth (d^-1)</td>
<td>0.5-3</td>
<td>0.32-3</td>
<td></td>
</tr>
<tr>
<td>Substrate affinity constant (K_{NH_4}) (mg N/L)</td>
<td>0.027-0.85</td>
<td>-</td>
<td>Koops &amp; Pommereining-Röser, 2001</td>
</tr>
<tr>
<td>Substrate affinity constant (K_{NO_2}) (mg N/L)</td>
<td>-</td>
<td>0.06-17</td>
<td></td>
</tr>
<tr>
<td>Oxygen affinity constant (K_{O_2}) (mg N/L)</td>
<td>0.03-1.6</td>
<td>0.3-2.5</td>
<td>Princic et al, 1998</td>
</tr>
<tr>
<td>pH range</td>
<td>5.8-8.5</td>
<td>6.5-8.5</td>
<td></td>
</tr>
<tr>
<td>Optimal pH</td>
<td>7.5-8.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dissolved Oxygen range (mg O_2/L)</td>
<td>3-4</td>
<td></td>
<td>Barnes &amp; Bliss, 1983</td>
</tr>
<tr>
<td>Optimal T (ºC)</td>
<td>25-30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Apart from the mentioned characteristics above and despite the basic metabolism appear to be more or less uniform within the different groups of AOB and NOB, it is possible to observe some ecophysiological differences between their representatives.

In aquatic environments, some AOB species occur attached to flocs or biofilms and appear to be self flocculating in pure cultures. Other species demonstrated to be able to exist both as individual planktonic cells or colonizing existing biofilms or flocs (Stehr et al, 1995).

Concerning NOB, *Nitrobacter* species occur as free-living cells while *Nitrospira* representatives have been observed attached to flocs or biofilms in their natural environments. Even in environments with a high amount of *Nitrobacter* organisms, only *Nitrospira*-like cells were found to be attached (Juretschko et al, 1998). In general AOB and NOB can be found in a wide variety of habitats including soils, rocks, fresh- and saltwaters and sediments.
1.7 **Tools in bacterial taxonomy**

The ranges of tools that can be used in order to study bacterial taxonomy can be divided in two main groups: genotypic methods and phenotypic methods.

Genotypic methods are directly related to DNA and RNA molecules and currently dominate modern taxonomic studies. Classification according to these methodologies should result from natural relationships as they are encoded by DNA. Phenotypic methods are not directly related to DNA or RNA and comprise classical phenotypic methods by analysis of morphological, physiological and biochemical features of cells. Other phenotypic non-classic approaches include numerical analysis, typing methods and chemotaxonomic techniques (Vandamme et al, 1996). Some relevant methodologies for this study are discussed below.

1.7.1 **Genotypic methods**

1.7.1.1 Gene sequencing

Because of it's presence in all bacteria, its constant function and the existence of highly conserved as well as more conserved domains, the rRNA operon is accepted as the best target for studying phylogenetic relationships establishing the major role of the relationships among nucleotide sequences in the definition of bacterial species. In this way, all species descriptions should include an almost complete 16S rRNA sequence (Vandamme et al, 1996; Stackebrandt, 2002).

Further studies showed some limitations of this technique in terms of taxonomic characterization. Several organisms sharing almost similar 16S rRNA sequences but low DNA-DNA hybridization values were found. Although 16S rRNA gene sequences analysis has proven to be useful in order to classify many bacterial groups, has also been showing limited variation between closely related taxa (Fox, et al, 1992). Description of new species should choose a polyphasic approach. According to this approach, classification is based on multiple genotypic and phenotypic methods to determine intra and interspecies relatedness (Stackebrandt, 2002).

In the case of AOB the *amoA* gene was combined with the 16S rRNA gene in order to increase the resolution. The gene *amoA* presents a higher level of sequence variation than the 16S rDNA, useful to discriminate closely related strains (Purkhold et al, 2000)
1.7.2 Phenotypic methods

1.7.2.1 Classical phenotypic approach

Classical phenotypic features of a bacteria include morphological, physiological and biochemical characteristics. Morphology is related to both cellular and colonial features. Physiological and biochemical data includes several types of measurements directly related to growth kinetics as a function of different variables (pH, temperature, salinity, substrate and others) (Vandamme et al, 1996).

This type of analysis requires standardized procedures to allow reproducibility. The Biolog system is a miniaturized biochemical test that was developed in order to characterize microbial communities, based on carbon source oxidation patterns. Direct incubation of whole environmental samples in Biolog plates may produce patterns as a function of the metabolic response of the community in the sample (Garland & Mills, 1991).

In chapter 2, several dilutions of two inocula are analysed in terms of their Biolog pattern.

1.8 Enhancement of nitrifiers activity by heterotrophic bacteria

Stable multispecies populations result from beneficial exchanges of substrates. Autotrophic nitrifiers reduce inorganic carbon to produce organic carbon present in cells. At the same time they release soluble microbial products (SMP) into solution that result from the substrate metabolism. Some data showed that the SMP products released by nitrifiers could provide a supplementary organic substrate for heterotrophic bacteria promoting their growth and stability.

In the presence of organic carbon, nitrifiers are normally outcompeted by heterotrophs due to their lower growth rate. However, in environments with low inputs of organic substrates (low ratio C/N) the production of SMP by nitrifiers was verified by measuring significant amounts of soluble COD in the output stream. Apparently, the amount of produced COD would be able to maintain a stable heterotrophic population (Rittman et al, 1994).

In the same way, the oxidizing activity of Nitrobacter is higher when the supplied media contain low amounts of organic matter. This effect could be explained either by the presence of low amounts of organic matter or by the stimulation of heterotrophic growth thanks to that organic matter. By applying a fermentation filtrate of Pseudomonas sp. it was possible to notice an increase not only in the rate of oxidation of nitrites but also in the growth of rate of Nitrobacter (Blanc et al, 1986).

In the same way, the bacterial composition of a carbon-limited autotrophic nitrifying biofilm fed only with TAN as energy source was analysed by fluorescence in situ hibridization (FISH). FISH analysis showed that this autotrophic biofilm was composed in 50% by heterotrophic bacteria. The others 50% were composed by nitrifiers in the same relative amount (Kindaichi et al, 2004).
More recently, by using a cloning-sequencing approach it was possible to identify a diverse heterotrophic community in a commercial nitrifying inoculum (Vanparys, 2006).

1.9 Nitrifying biofilters

In closed or semi-closed aquaculture or aquaria systems some type of treatment must be installed in order to assure water quality levels. The most common problems arise from TAN, organic matter and CO₂ accumulation and low levels of dissolved oxygen (Muir, 1982). Only biological processes are economically viable when applied in the industrial scale. TAN removal can be obtained by stripping, ion-exchange resins or by oxidation with chlorine. However, all these processes are costly or involve toxicity risks (Blanc, 1986). Biological removal with an appropriate inoculum and a suitable support matrix in order to stimulate biofilm development is commonly applied in both small and large scale processes. The most common type of biofilter consists of a submerged matrix over which water flows (Wheaton et al, 1994).

Concerning aquaria systems it is possible to apply the same support in either freshwater or saltwater. The critical steps to successfully establish a population of nitrifiers are normally the existence of solid support with good aeration and a certain amount of TAN or nitrite as energy source (Hovanec et al, 1996).

For newly set-up aquaria, it’s possible to notice TAN or nitrite accumulation before maturation of the biofilter. Some studies reported start up times up to 60 days (Carmignani and Bennett, 1977). As a result of the accumulation of several toxic metabolites in newly set up aquaria, a high fish mortality rate can be observed when no stable bacterial community is yet established. Given the slow growth rates verified among the various species of nitrifiers, addition of commercial nitrifying inocula can therefore be used in both aquaculture systems and aquaria in order to reduce the start-up period of a biofilter and thus, prevent the commonly known “new tank syndrome”.

Previous studies reported different results on the effect of commercial microbial products in aquaculture ponds. Regarding nitrifying inocula, King (1986) observed increase TAN removal from pond effluents. Other studies reported that biofilters seeded with commercial inocula were found to increase the rate of nitrification significantly when compared with unseeded biofilters (Labomascus, 1987; Matias et al, 2002). Timmermans and Gerard (1990) conclude that commercial inocula were ineffective for unknown reasons.

In general, commercial preparations range from pure cultures of *Nitrosomonas* and *Nitrobacter* or *Nitrospira* sp. to mixtures of both types of nitrifiers and to cultures that also combine heterotrophs with nitrifiers. Hovanec et al (1996) suggested that the failure of some commercial preparations could be explained by the use of inappropriate species. In aquaria, the establishment of *Nitrospira*-like bacteria coincided with the start of nitrite oxidation. After
inoculation with commercial preparations containing *Nitrobacter* sp., some days later a stable community of *Nitrospira* appeared to be formed (Hovanec et al, 1996). In contrast with this study and by using specific primers for *Nitrospira*, no amplicons were obtained in both saltwater and freshwater aquaria biofilters. However, it was possible to yield some *Nitrobacter* oriented amplicons (Grommen, 2005). After analysis of the biofilm structure from an aquaculture filter it was also possible to notice evenly distributed *Nitrobacter* colonies. *Nitrospira* sp. generally prefers low nitrite concentrations and in a certain way, nitrite concentration could be the major variable controlling the competition between *Nitrospira* and *Nitrobacter* (Spieck, 2006).

Concerning AOB it seems clear that *Nitrosomonas marina*-like are the bacteria most responsible for ammonia oxidation in aquaria (Burrel, 2001). Despite their lower activity, bacteria related to the *Nitrosospira* cluster and *Nitrosomonas europaea*-*Nitrosococcus mobilis* cluster were also detected in freshwater biofilters (Grommen, 2005). In general *Nitrosomonas* sp. are the most common type of ammonia oxidizers found in halophilic environments and wastewaters while *Nitrosospira* are mainly spread along soil and freshwater (Princic, 1998).

### 1.10 Commercial nitrifying inocula (NI)

A wide variety of commercial nitrifying inocula can be found. Little studies on these products are known. However, one commercial inoculum called Ammonia Binding Inoculum Liquid (ABIL) has been extensively studied (Grommen et al, 2002; Rombaut et al, 2003; Grommen et al, 2005; Vanparys et al, submitted). This inoculum is obtained by selective enrichment of the nitrifying community that can be found in activated sludge. The enrichment is made by using an autotrophically fed reactor. It was possible to show that this product was able to avoid ammonia and nitrite accumulation in both freshwater and saltwater aquaria (Grommen et al, 2002; Grommen et al, 2005).

A detailed study on the bacterial community of this inoculum was also made. Apart from the limited diversity of nitrifiers it was possible to find a diverse heterotrophic community. Among the nitrifying community *Nitrosomonas aestuarii* and *Nitrobacter winogradsky* related strains were found (Vanparys, submitted).

In the second chapter, four different commercial inocula and one product in the test phase were studied in terms of their specific activity in freshwater. Both TAN and nitrite oxidation were determined.

### 1.11 Patents

A patent is a set of exclusive rights granted by a state to a person or organization for a fixed period of time. These exclusive rights include the right to decide who may use the invention
that is object of the patent. The owner(s) of a patent may license other parties to use the invention or sell its rights. Once a patent expires the invention enters the public domain, becoming available to commercial exploitation by others.

When protected by a patent, an invention can not be commercially made, used, distributed or sold without the patent owner’s permission (WIPO, 2007).

There is no global definition in which kind of inventions can be patented. In most of the countries, scientific theories, mathematical methods, plant or animal varieties, discoveries of natural substances, commercial methods, or methods for medical treatment (as opposed to medical products) can not be patented. However, other countries grant patents that protect these types of inventions. For instance, a less restrictive law can be found in the USA, where only laws of nature, physical phenomena, and abstract ideas are not patentable (USPTO, 2007).

Patents that are related to an invention or a discovery in biology have been object of controversy since the first patent application on a single strain of *Pseudomonas* was presented in 1972. In USA since 1980, some type of living organisms in which bacteria are included can be patented as long as they incorporate human intervention or as it was concluded by court “anything under the sun that is made by man” is patentable (Stix, 2006).

In 1998, the European Parliament approved the directive 98/44/EC on the “patentability of biotechnology”. This directive states in its third article that inventions which are new, involve an inventive step and are susceptible of industrial application “shall be patentable even if they concern a product consisting of or containing biological material or a process by means of which biological material is produced, processed or used”. The same article states also the patentability of biological material “isolated from its natural environment (...) even if it previously occurred in nature” (EP, 1998). At the time of its approval the passage was controversial and remains so today. In July 2003 the European Commission referred Germany, Austria, Belgium, France, Italy, Luxembourg, the Netherlands and Sweden to the Court of Justice of the European Communities for their failure to transform the Directive into their national patent laws by July 2002 (Palombi, 2003).

Beyond this discussion on the patentability of biotechnology, an invention must include always an inventive step and be of practical use.

Despite including elements that are already known, an invention to be considered as so will have a new characteristic that is not known in the sum of existing knowledge. The sum of existing knowledge is named as “prior art”. Prior art is also known as “state of the art” and refers to all the information that has been made available to the public. Already commercialized inventions integrate the state of the art and as a consequence of that, similar inventions can not be patented (WIPO, 2007).

Currently there is no possibility for grant a patent simultaneously in several countries. As each country has its patents legislation, several filings have to be made to seek for a patent.
protection in each country individually. Some treaties were established in order to allow regional filing. The Patent Cooperation Treaty (PCT), the European Patent Convention (EPC) and others, give the possibility to apply for a patent in different countries by filling just one document. Later, several requirements have to be fulfilled in order to grant a patent in each specific country.

Apart from patents, also utility models provide invention protection. There are few differences between patents and utility models. The later are generally cheaper to obtain and to maintain and the requirements to acquire them are less stringent than for patents. At the same time the term of protection is shorter for utility models. Currently only 46 countries provide utility model protection (WIPO, 2007).

The public document that provides all the necessary data about a patent is called a patent specification. The patent specification must describe in a complete and clear way the invention as long as one skilled person could carry out the described invention. Information regarding industrial applicability, the technology that supports the invention, the prior art considered useful for understanding the invention and practical examples (including drawings) must be included in the patent specification.

Probably the main part of the patent specification is dedicated to the claims. Claims have legalistic nature in the way they translate the invention into something that can be object of legal procedures. Each claim should be written as a single concise sentence without being narrow or broad. Narrow claims could make easy for a competitor to benefit from the invention without infringing the claims. On the opposite side, broad claims could result in a lack of newness given the fact they would be including too much prior knowledge (MyIPO, 2005).

### 1.11.1 Products

According to Kotler et al (2006) a product is something that can be offered to a market in order to satisfy a want or need. Market is understood as any type of interface that allows buyers and sellers to change information in order to carry out the exchange of goods and services. A product line consists in a group of closely related products as they are sold to the same customer groups, function in a similar manner, have similar prices or distributed by using the same type of channel (Kotler et al, 1989).

In the present study, products from the same line should be understood as something that is sold to the same group of customers and therefore have similar purposes.

In the second chapter, thirteen patents and one utility model are analysed. This analysis aims to understand in which ways the existing granted protection could inhibit the production and commercialization of new products from the same product line. Despite having no legalistic
nature, several publications cited in this text also constitute the body of existing art related to the considered product line. In the same way, even if a certain patent does not exclude others from commercializing an invention it will always integrate the state of the art avoiding in that way the arising of new hypothetical patents.

1.12 Activated Sludge

Activated sludge is currently the most widely used biological treatment process for both domestic and industrial wastewaters (Wanner, 1994). This type of treatment refers to a biological process that uses a mixed community of organisms that metabolise and transform organic and inorganic substances into environmentally acceptable substances. Typically, activated sludge is constituted by approximately 95% bacteria and 5% higher organisms such as protozoa, metazoan, fungi, rotifers, etc (Seviour and Blackall, 1999).

The three primary nutrients that must be removed from effluents in order to prevent decline of water bodies are carbon, nitrogen and phosphorous. As the removal of nitrogen and phosphorous involve heterotrophic conversions requiring an electron donor, effluents with low COD/N or COD/P ratios experience difficulties in removing residual nitrogen or phosphorous. The typical activated sludge system is generally characterized by two tanks placed in series.

In the first tank, the wastewater is mixed with a consortium of organisms growing in the form of aggregates or microbial flocs. The pollutants are metabolised by the organisms or entrapped in the aggregates (Wanner, 1994). This tank was usually called aeration tank. This designation is being less and less used since today several modifications to the typical activated process were performed. Removal of nitrogenous pollutants has its basis in the activated sludge process and is several times accomplished under anaerobic or anoxic conditions.

Denitrification takes place in anaerobic conditions in the absence of oxygen and nitrate, when performed by autotrophic bacteria or anoxic conditions in the presence of nitrate, without oxygen, when is performed by heterotrophic bacteria.

The second tank is called clarifier. In this tank, the mixed liquor (mixed water and biomass) is then separate into clear water and biological sludge. Normally there is also a recycling system to return back, part of the sludge that settled (Wanner, 1994).

Given activated sludge’s microbial diversity, consequence of the several substrates that can be found in effluents, samples of aerobic activated sludge from different facilities are used in chapter 1. These samples are used as representative of a diverse heterotrophic community.

In chapter 3, an enrichment was obtained starting from activated sludge.

Usually, complete nitrogen removal can be achieved with a ratio C/N of 7 to 1 (Pochana and Keller, 1999). However, many types of wastewater have a high TAN and a low carbon content, resulting in C/N ratios even lower than 1.

Conventional nitrification-denitrification processes are not effective given this type of effluents.
1.13 **Denitrification**

Nitrification is an oxygen-requiring process and therefore requires an aerobic environment. During the subsequent denitrification step, nitrate or nitrite are converted to nitrogen in anoxic conditions, allowing return of the gas back to the atmosphere. In aquaria, nitrate tends to accumulate as the final product of the nitrification process. However, acute toxicity of nitrate to fish is very low with LC$_{50}$ values ranging between 1300 – 1500 mg N-NO$_3^{-}$/L (Scott and Crunkilton, 2000). In domestic aquaria, nitrate control is made by regular water changes but this process can not be applied in large aquaculture systems. Several bacteria distributed among more than 50 genera are able to denitrify, including chemoorganotrophic, lithoautotrophic and phototrophic bacteria and some fungi (Zumft, 1992). Concerning aquaculture, methanol can be used as an electron donor since its consumption does not generate organic acids that could be toxic for fish (Lee et al, 2000). Four enzymatic reactions are involved in the denitrification process, catalysed by four metallo proteins: nitrate reductase (Nar), nitrite reductase (Nir), nitric-oxide reductase (Nor) and nitrous oxide reductase (Nos). Nitrite reductase forms the distinguishing feature between denitrifiers and nitrate reducers (Vanparys, 2006).

1.14 **Anoxic ammonia oxidation (anammox) by anoxic ammonia-oxidizing bacteria (AnAOB)**

Anoxic ammonia oxidation (anammox) is the microbiological process by which TAN and nitrite are converted to dinitrogen gas in the absence of organic carbon. Broda (1977) was the first predicting the existence of chemolithoautotrophic bacteria capable of anoxic ammonia oxidation, by comparing the Gibbs energies of the aerobic ($\Delta G = -118$ kJ/mol) and anoxic ($\Delta G = -357$ kJ/mol) ammonia oxidations. In the early 1990s it was observed in a denitrifying pilot plant (Gist Brocades yeast factory, Delft, Netherlands) that the anoxic removal of ammonia and nitrate was accompanied by dinitrogen gas formation. This was the first experimental confirmation of anammox (Mulder et al, 1995). The following equation with ammonia as electron donor for nitrate reduction was proposed (Mulder et al, 1995):

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+$$  \hspace{1cm} (10)

Later it was observed that ammonia was oxidized with nitrite instead of nitrate (van de Graaf et al, 1995):
Based on mass balances for enrichment cultures, anammox stoichiometry was presented as (Strous et al 1998):

\[
NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O
\]  

Although the pathway remains unknown, anammox bacteria fix CO\textsubscript{2} as the major carbon source for its growth. At the same time a small part (11\%) of the nitrite is also oxidized to nitrate.

After enrichment, anammox bacteria were successfully purified by density gradient centrifugation. It was possible to show that the purified culture (99.6\% pure) was able to use ammonia and nitrite to produce nitrogen gas. By PCR amplification of the 16S rDNA sequence with a universal primer set it was found that anammox bacteria belonged to the family of the planctomycetes. This anoxic ammonia-oxidizing organism was named “\textit{Candidatus Brocadia anammoxidans}” (Strous et al, 1999).

After 16S rDNA sequencing, specific oligonucleotide probes for application in FISH were designed. \textit{Brocadia anammoxidans} and the related “\textit{Candidatus Kuenenia stuttgartiensis}” were found to be present in many wastewater treatment systems around the world (Schmid et al, 2000). Currently, anammox bacteria are assigned to three different genera: \textit{Candidatus “Brocadia”, Candidatus “Kuenenia”} and \textit{Candidatus “Scalindua”} (Figure 1.5).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_5.png}
\caption{Phylogeny of planctomycetes responsible for anammox (Strous and Jetten, 2004).}
\end{figure}

All the three genera have the same metabolism and share a similar ultrastructure characterized by the presence of an anammoxosome. In common with other planctomycetes, AnAOB also
have a proteinaceous cell wall without peptidoglycan. The chromosome can be found in a distinct nucleus membrane separated by the cytoplasm (Schmidt, 2001). The anammoxosome is surrounded by a dedicated membrane consisting of ladderane lipids and can be separated from the cell. Considering the suggested biochemical pathway of anammox (Damsté et al, 2002), the proton motive force is generated over the anammoxosome membrane via separation of charges (Figure 1.6).

In Table 1.5, some of the most important physiological parameters for the AnAOB are shown. The growth rate (doubling time of 11 days) and the growth yield (0,11 g VSS/ g N-TAN) are both very low what in practical terms can represent an advantage since the sludge production is also low.

---

**Table 1.5** Summary on some physiological parameters of AnAOB.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AnAOB</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generation time (d)</td>
<td>11</td>
<td>Jetten and Strous, 1999</td>
</tr>
<tr>
<td>Specific Growth (d⁻¹)</td>
<td>0,065</td>
<td></td>
</tr>
<tr>
<td>Growth Yield (g VSS/g N-TAN)</td>
<td>0,11</td>
<td></td>
</tr>
<tr>
<td>TAN affinity constant (mg N/L)</td>
<td>20-40</td>
<td>Schimdt, 2003</td>
</tr>
<tr>
<td>Nitrite affinity constant (mg N/L)</td>
<td>30-40</td>
<td>van de Graaf et al, 1996</td>
</tr>
<tr>
<td>pH range</td>
<td>6,4-8,3</td>
<td>Strous et al, 1999</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Maximum Dissolved Oxygen (mg O₂/L)</td>
<td>0,064</td>
<td>Jetten, 2001</td>
</tr>
<tr>
<td>Optimal T (ºC)</td>
<td>40</td>
<td>Jetten and Strous, 1999</td>
</tr>
<tr>
<td>Maximum nitrite concentration (mg N/L)</td>
<td>90-180</td>
<td>Jetten and Strous, 1999</td>
</tr>
<tr>
<td>Maximum phosphate concentration (mg P/L)</td>
<td>60</td>
<td>van de Graaf et al, 1996</td>
</tr>
<tr>
<td>T range (ºC)</td>
<td>20-43</td>
<td>Strous et al, 1999</td>
</tr>
</tbody>
</table>
1.15 Conventional nitrification-denitrification processes for wastewater treatment

Deterioration of quality of inland and coastal waters is a serious environmental problem. Wastewaters containing nitrogen and phosphorous is of particular concern.

Regarding wastewater treatment, TAN removal is generally achieved by a combination of nitrification and denitrification (Focht and Verstraete, 1977). However, conventional nitrogen removal processes used in low nitrogen loaded sewage can not be applied to treat nitrogen-rich wastewaters since its extremely high expenses for aeration and the need of exogenic COD supply.

Biological nitrogen removal is effective and inexpensive but the autotrophic micro-organisms involved in the process grow slowly. At the same time, operational control of aerobicity or anaerobicity of the process is complex. In order to deal with these limitations several types of bioreactors have been studied to improve the efficiency of nitrogen removal processes.

Conventional nitrification-denitrification is based on autotrophic nitrification and heterotrophic denitrification. Heterotrophic denitrification occurs with several possible electron donors, including methanol, acetate, ethanol, lactate and glucose. As these two steps are carried out in different conditions and by different micro-organisms they have to occur physically or chronologically separated. Nitrification reaction consumes 4.2 g O$_2$ for each gram of N-TAN nitrified. During the denitrification process, the carbon requirement is equal to 2.47 g methanol per gram of N-NO$_3^-$ making its utilization quite expensive (Khin and Annachhatre, 2004).

Some intermediate compounds produced and excreted by methanotrophs can be used as carbon sources in denitrification. Downstream integration of anaerobic digestion with methane producers and denitrifiers could be a feasible approach in order to provide denitrifiers suitable electron donors (Khin and Annachhatre, 2004).

As a consequence of the low organic carbon content of wastewater, good removals in effluents with high nitrogen concentrations are difficult to achieve, thus many WWTP do not meet the discharge standard of 10 mg N/L (Jetten et al., 2002).

After discovering the anoxic oxidation of ammonia by planctomycetes and more recently, the anoxic metabolism by the better known ammonia oxidizers, new possibilities concerning nitrogen removal have been developed.

At the same time and even without the intervention of anammox organisms recently discovered it was possible to change the typical approach that the only possibility to remove nitrogen from wastewater would be the complete oxidation of ammonia to nitrate followed by heterotrophic denitrification.
1.16 New processes for nitrogen removal

Various novel biological processes such as the single reactor system for high ammonia removal over nitrite (SHARON), the Anammox process, the completely autotrophic nitrogen removal over nitrite (CANON) and the oxygen-limited autotrophic nitrification/denitrification (OLAND) have been exclusively developed for nitrogen removal. From these set of approaches, only “pure” SHARON is carried out without anammox bacteria. Partial nitrification to nitrite is a critical step for implementing these technologies as nitrite is required as substrate or intermediary compound (Philips et al, 2002). The introduction of anammox as an organism able to perform the removal process from the combination of TAN and nitrite would replace the conventional denitrification process, saving costs during the nitrification process apart from several other advantages (Strous and Jetten, 2004). The coupled process SHARON-Anammox has been inspiring other approaches such as CANON (Hao, 2001) and OLAND (Kuai and Verstraete, 1998), by combining partial nitrification and Anammox reaction in just one reactor.

1.16.1 SHARON

SHARON was the first process in which nitrification/denitrification with nitrite as an intermediary compound has been achieved under stable conditions and used in full-scale operations (Mulder et al, 2001). The process was developed at the Delft University of Technology (Hellinga et al 1998). As the process is especially suitable for waste waters with a low ratio C/N, wastewaters from composting processes and sludge drying, landfill leachates and wastewater from digestion of organic waste and manure are candidates to be treated by this process.
The SHARON process is operated without biomass retention in a single reactor at relatively high temperature (35°C) and pH > 7 (Hellinga et al, 1998).

Figure 1.8 Left: By eliminating the nitrite oxidation to nitrate and the subsequent step of nitrate reduction (dotted line) it’s possible to save up to 40% and 25% respectively in terms of COD and aeration. Right: Given a temperature higher than 15 ºC, the minimum residence time (MRT) in order to avoid wash-out, is always lower in the case of AOB. This process takes advantage from the different growth rate that exists between AOB and NOB.

At high temperatures, AOB have a significant higher growth rate. By controlling the aerated retention time to about 1 day while operating at relatively high temperatures and without sludge retention, NOB are washed out of the reactor (Figure 1.8, Right). Consequently the oxidation reaction stops at nitrite (Figure 1.8, Left) (Hellinga et al, 1998). As the process depends on high temperatures, it’s not suitable to every type of wastewaters. However, many wastewaters with high TAN concentrations also have a high temperature, such as sludge liquor (Schmidt et al., 2003).

Aeration in this particular case is not only necessary for oxygen supply but also to strip CO₂ from the reactor in order to control the pH. Methanol is the most commonly used electron donor being dosed periodically while the aeration is switched off.

The oxidation of TAN is responsible for the pH decrease and in face of this, pH control is critical. At pH values close to 6.5, concentration of free ammonia does not allow sufficient growth of AOB. At the same time, high pH values are favourable to AOB that grow faster than NOB in these conditions. However, increasing concentrations of free ammonia can also be inhibitory for AOB (Anthonisen et al, 1976). Since NOB have lower affinity for DO, a low DO is a restraint for its growth (Hellinga et al, 1998).

By stopping the oxidation at nitrite it’s possible to save 25% in terms of aerations costs and 40% in terms of exogenous COD that would be added (Figure 1.8, Left).

1.16.2 AOB in association with AnAOB: 2 reactors
(SHARON+Anammox) and 1 reactor (OLAND and CANON)

1.16.2.1 SHARON + Anammox

As previously seen (1.14), the Anammox process corresponds to a denitrification of nitrite with ammonia as electron donor. Given a wastewater stream with a high TAN concentration, the
coupled process SHARON-Anamnox will need a preceding partial nitrification step in order to convert half of the ammonia to nitrite and thus creating the appropriate stoichiometric mixture. By removing the anoxic periods usually applied to SHARON without adding methanol it is possible to yield the desired TAN/nitrite mixture according to following equation:

\[
NH_4^+ + HCO_3^- + 0,75O_2 \rightarrow 0.5NH_4^+ + 0.5NO_2^- + CO_2 + 1,5H_2O \quad (13)
\]

After converting approximately 50% of the TAN, the decrease of the pH will avoid oxidation of the remaining TAN (Schmidt et al, 2003). In this way the effluent from this “half”-SHARON process will suit as influent for the Anammox process where TAN and nitrite are anoxically converted to dinitrogen gas and water.

This process does not require any addition of exogenous COD. In face of this, COD and nitrogen removal operations in a wastewater treatment plant, can be optimized separately without making complex compromises between COD and nitrogen removal that are currently observed when the conventional nitrification-denitrification approach is present (Jetten et al, 1997).

Approximately 75% of the total nitrification aeration costs are spent in order to convert TAN to nitrite. Compared to the conventional process (1.15), coupled SHARON-Anamnox process will oxidize to nitrite only 50% of the available TAN, thus saving 62.5% of the aeration costs and 100% in terms of exogenous carbon source (there is no need to supply an additional carbon source). At the same time, the combined process consumes CO\textsubscript{2} and it is 90% less expensive (Jetten et al, 1997).

1.16.2.2 CANON

As SHARON process successfully combines the aerobic step of autotrophic nitrification with the anoxic process of heterotrophic denitrification, also CANON combines partial nitrification and anammox in a single, aerated reactor (Third et al, 2002).

The CANON process is based on the stable interaction between Nitrosomonas-like AOB and Planctomycete-like anoxic ammonia-oxidizing (Anammox) bacteria. AOB oxidize ammonia to nitrite consuming oxygen and thus, creating anoxic conditions ideal for anammmox. Oxygen concentrations higher than 0.5 mg/L have no effects in terms of TAN oxidation as expected. However, NOB are outcompeted by AOB in these conditions.

The process must be applied under oxygen-limited conditions (0.4 -1.0 mg/L at 20°C) (Third, 2002). As just one reactor is required, the economics could be an advantage concerning full-scale applications of the process.

After conversion of ammonia to nitrite by AOB, anammox bacteria will convert TAN and the produced nitrite to dinitrogen gas and a small amount of nitrate. The CANON process has been carried out in a gas lift reactor. A high ammonia removal rate was achieved (1.5 kg/(m\textsuperscript{3} reactor.d)), 20 times higher as compared to the previously lab tests with an SBR (Sliekers et al, 2003).
1.16.2.3 OLAND

Through the OLAND process, NH$_4^+$ is autotrophically oxidized to dinitrogen gas by using NO$_2^-$ as electron acceptor under oxygen-limited conditions, consuming 63% less oxygen and 100% less exogenous COD. This process was first described for a mixed culture previously enriched from activated sludge obtained in a wastewater treatment plant, operating in a sequential batch reactor (SBR) (Kuai and Verstraete, 1998).

Kuai and Verstraete (1998), described the OLAND process in a RBC for a mixed culture of normal nitrifiers dominated by AOB. The mixed culture used as an inoculum was a normal nitrifying sludge fed with ammonium and mineral nutrients. At the same time, during the operation of the OLAND system, the composition of the feed was rather similar to the feeding of the inoculums’ breeding reactor.

A different OLAND system was started up by using seed sludge from a methanogenic reactor. Afterwards, the formed biofilm was analysed in more detail and found to be dominated by two major groups of bacteria. The mature biofilm was thus found to be constituted by AOB that convert ammonium to nitrite with oxygen as electron acceptor and AnAOB (close relative of *Kuenenia stuttgartiensis*) that subsequently oxidize ammonium with nitrite as the electron acceptor (Pynaert et al, 2003, 2004).

The Figure 3.1 presents a general scheme of the mature OLAND biofilm in a RBC. The following equations summarize the overall N removal stoichiometry:

![Figure 1.9 General scheme of a mature biofilm developed in a OLAND reactor. This structure results from a gradual adaptation process (Vlaeminck, 2005, adapted)](image)
AOB: $1,32NH_4^+ + 1,98O_2 \rightarrow 1,32NO_2^- + 1,32H_2O + 2,64H^+$  \hspace{1cm} (14)

AnAOB: $NH_4^+ + 1,32NO_2^- + 0,13H^+ \rightarrow 0,26NO_3^- + 1,02N_2 + 2,03H_2O$  \hspace{1cm} (15)

OLAND: $2,32NH_4^+ + 1,98O_2 \rightarrow 0,26NO_3^- + 1,02N_2 + 2,51H^+ + 3,35H_2O$  \hspace{1cm} (16)

OLAND (1 mol NH$_4^+$): $NH_4^+ + 0,85O_2 \rightarrow 0,11NO_3^- + 0,44N_2 + 1,14H^+ + 1,44H_2O$  \hspace{1cm} (17)

Under a high TAN loading rate, the biofilm will evolve as a function of time in terms of its composition. NOB are likely to be present in the biofilm, specially in an early stage of its development. However NOB are not desired since they are able to oxidize nitrite to nitrate when enough oxygen is present. In this way, the total nitrogen content in the effluent in different forms can be higher. AnAOB and NOB compete for nitrite, but only the first are able to remove nitrogen from the effluent, converting it into N$_2$. At the same time, also AOB and AnAOB will compete for TAN (Windey et al, 2005; Pynaert, et al, 2003). However, these organisms convert the substrate in fundamental different conditions and probably the spatial arrangement in the biofilm allows a stable coexistence between both. Apart from oxidizing the TAN content of the effluent, AOB bacteria are also able to promote an anoxic environment in the inner part of the biofilm creating a suitable environment for AnAOB. The absence of NOB in a mature biofilm could be explained by their lower affinity for oxygen compared to AOB. In an oxygen limited environment, NOB will grow in the outer part of the biofilm, increasing their vulnerability to wash-out, decreasing in number till an eventual absence (Pynaert et al, 2003).

1.16.3 NO$_x$ process

In the presence of NO$_x$, *Nitrosomonas*-like organisms are able to nitrify and denitrify simultaneously even in aerobic conditions. Dinitrogen gas is the main product and about 40% of the total TAN load is converted to nitrite.

NO$_x$ (NO/NO$_x$) is used as a regulatory signal that induces denitrification activity of AOB when added in trace amounts (200-1000 ppm). As a consequence, approximately 50% of the generated reducing equivalents are transferred to nitrite instead of oxygen, saving 50% of the total oxygen demand that would be needed to finish the process.

Some studies on a pilot plant showed that ammonia oxidation activity was strongly dependent on the NO$_x$ concentration.

The remaining nitrite can be removed in a small denitrification step by using methanol as electron donor. In order to complete the process it’s possible to reduce the amount of exogenous COD in about 80% and the supply of oxygen in 50%.
Table 1.6 Comparison of the processes described above in terms of number of reactors, aeration conditions, exogenous COD and oxygen savings, nitrogen removal and bacteria involved (Jetten et al, 2002; adapted).

<table>
<thead>
<tr>
<th>Process Description</th>
<th>Number of Reactors</th>
<th>Conditions</th>
<th>COD saving</th>
<th>Oxygen saving</th>
<th>N-removal</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Conventional nitrification + denitrification</td>
<td>2</td>
<td>aerobic-anoxic</td>
<td>0%</td>
<td>0%</td>
<td>95%</td>
<td>AOB+heterotrophs</td>
</tr>
<tr>
<td>B) Nitritation + Denitrification (Partial Nitrification + Denitrification)</td>
<td>2</td>
<td>aerobic-anoxic</td>
<td>40%</td>
<td>25%</td>
<td>90%</td>
<td>AOB+heterotrophs</td>
</tr>
<tr>
<td>C) SHARON</td>
<td>1</td>
<td>aerobic-anoxic</td>
<td>40%</td>
<td>25%</td>
<td>90%</td>
<td>AOB + Heterotrophs</td>
</tr>
<tr>
<td>D) 1/2 SHARON + Anammox</td>
<td>2</td>
<td>aerobic-anoxic</td>
<td>100%</td>
<td>63%</td>
<td>90%</td>
<td>AOB + AnAOB</td>
</tr>
<tr>
<td>E) OLAND/CANON</td>
<td>1</td>
<td>oxygen-limited</td>
<td>100%</td>
<td>63%</td>
<td>90%</td>
<td>AOB+AnAOB</td>
</tr>
<tr>
<td>F) NO₃ process + denitrification</td>
<td>2</td>
<td>aerobic-anoxic</td>
<td>80%</td>
<td>50%</td>
<td>95%</td>
<td>AOB + heterotrophs</td>
</tr>
</tbody>
</table>

Figure 1.10 Flux diagrams of the different processes described above. A) Conventional nitrification-denitrification process; B) Nitrification over nitrite plus heterotrophic denitrification ("SHARON" in two reactors); C) Nitrification over nitrite plus heterotrophic denitrification (SHARON); C) Nitrification over nitrite plus anoxic ammonia oxidation ("half-SHARON" plus anammox); E) Nitrification over nitrite plus anoxic ammonia oxidation in the same reactor (OLAND and CANON); E) In the presence of gaseous nitrogen dioxide, denitrification activity of AOB is induced (Schimdt et al, 2003; adapted).
2 Study of commercial nitrifying inocula

Abstract

The ammonia concentration in aquaria or aquaculture systems was found to be the most important factor limiting fish density. In order to recirculate a suitable effluent for use in aquaria or aquaculture systems, removal of ammonia by biological activity appears to be a cost-effective methodology.

In this study, four commercial nitrifying inocula (NI) plus one NI in the test phase were tested concerning their total ammonium nitrogen (TAN) and NO$_2^-$ (nitrite) oxidation activities. Tests were performed in freshwater with initial TAN and NO$_2^-$ concentrations up to 40 mg N/L. Two of these commercial NI (JBL and BioSpira) showed no visible activity. It was possible to observe low activity concerning two other products (Product A and Baktinetten) but only one of the tested nitrifying suspensions (ABIL) showed good activity concerning both TAN and NO$_2^-$ oxidation. In particular, the maximum reported activities for ABIL were respectively, for TAN and NO$_2^-$, 0.644 ± 0.018 g N-TAN/g VSS.d and 0.313 ± 0.052 g N-NO$_2^-$/g VSS.d.

Influence of phosphorous and the addition of several TAN pulses on the activity was also investigated for ABIL. It was possible to conclude that both factors are able to increase the activity of ABIL.

This NI was later tested in terms of its ability to degrade carbon sources (CS). Different CS were successfully degraded by ABIL with even higher activities than aerobic activated sludge (AAS) used as a control. These results show the diverse heterotrophic community accompanying the autotrophic bacteria present in the nitrifying suspension. The oxygen uptake rate (OUR) after addition of glucose and TAN was also determined for ABIL and AAS. For the TAN oxidation process, an OUR of about 12.1 mg O$_2$/g VSS.h was obtained for ABIL.
A concentração de amónia em aquários domésticos ou em sistemas de aquacultura é o factor mais relevante a limitar a densidade de peixe que é possível obter.

Por forma a recircular um efluente passível de ser utilizado na criação de peixes, a remoção de amónia através da actividade biológica apresenta-se como uma via eficaz e de baixo custo.

Neste estudo, quatro inóculos nitrificantes (IN) já em comercialização e um inóculo na fase de testes foram estudados, tendo em conta as suas actividades de oxidação de amónia e nitrito.

Os testes foram efectuados em água doce com concentrações iniciais de nitrito e TAN (total ammoniacal nitrogen) até 40 mg N/L.

Para dois dos IN testados (JBL and BioSpira) não foi possível observar qualquer actividade. Para o produto na fase de testes e um dos IN (Produto A e Baktinetten) foram registada baixas actividades. Apenas um dos IN (ABIL) mostrou uma boa actividade tanto em termos de oxidação do TAN como de nitrito. Em concreto, os valores máximos de actividade registados para este inóculo foram de 0.644 ± 0.018 g N-TAN/g VSS.dia e 0.313 ± 0.052 g N-NO$_2$-g VSS.dia.

A influência do fósforo e da adição de vários pulsos de TAN foi igualmente investigada para o ABIL. Foi possível concluir que tanto um factor como o outro exercem uma influência positiva na actividade medida.

Mais tarde, este inóculo foi testado em termos da sua capacidade para degradar diferentes fontes de carbono (FC). Efectivamente, várias FC foram degradadas pelo ABIL com actividades superiores aquelas verificadas para as lamas activadas utilizadas como controle. Este resultados demonstram a diversa comunidade heterotrófica que acompanha as bactérias nitrificantes que se encontram no IN. A taxa de consumo de oxigénio após adição de glucose e TAN foi também determinada para o ABIL e lama activada proveniente de sistemas aeróbios. Relativamente à oxidação de TAN, foi registada uma taxa de consumo de oxigénio de cerca de 12.1 mg O$_2$/g VSS.h.

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**Palavras-chave:** Nitrificação; Inóculo; Patente; *Nitrosomonas*; *Nitrobacter*; Amónia/Amónium; Nitrito; AOB; NOB

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**2.1 Introduction**

The total ammonium nitrogen (TAN) concentration in aquaria or aquaculture systems appears to be the most important factor limiting fish density. In water, ammonia exists in equilibrium with the ammonium ion. The sum of the two species is generally indicated as total ammonium nitrogen (TAN). The equilibrium between ammonia and its protonated form is strongly
dependent on pH and temperature. At pH 6, TAN concentration of 10 ppm will not produce any negative effect in fish but the same concentration at pH 7 is already considered toxic. Fish excrete ammonia and ammonium by the gills and also through skin and faeces as the main product from protein metabolism. Mineralization of feed residues also contributes for the increase of TAN in water.

Biological autotrophic conversion of ammonia to nitrate occurs through a two-step process called nitrification. In the first part of the process, ammonia oxidizing bacteria (AOB) convert ammonia to nitrite. Nitrite is subsequently converted to the relative harmless nitrate by nitrite oxidizing bacteria (NOB). Utilization of aquaria or aquaculture biofilters colonized by these autotrophic bacteria can effectively decrease TAN concentration and thus, produce a suitable environment for fish. Given the very slow growth rate of these organisms, long start-up times were reported for this type of systems.

Several types of commercial nitrifying inocula (NI) are currently available in the market, with the purpose to shorten the start up time of these biofilters. While some studies showed the poor efficacy of some preparations, other reported successful applications of the same. During the last ten years, several patents have been granted in order to protect some of these commercial preparations. However, only a few number of companies showed interest in granting patents concerning NI. Several studies predict the current increasing trend of the aquaculture business and the ornamental fish industry, bringing into question the need of developing new products and protect the same by patenting.

In this study, a patents survey was carried out with the aim of giving some insights concerning the major players in the market, the type of protection the current patents are providing and the possibilities of patenting new products. Some commercial NI were also tested in order to determine specific TAN and NO\textsubscript{2}\textsuperscript{-} oxidation activities.

More tests were performed with one NI in particular called ABIL. Influence of phosphorous in the nitrification activity as well as the effect of adding several TAN pulses on the activation of the inoculum were investigated. The ability of this NI to oxidize different carbon sources (CS) as well as the typical oxygen uptake rates (OUR) of the same were also studied.
2.2 Materials and methods

2.2.1 Patents survey

A patents survey was conducted based solely on internet research during March 2007. Esp@cenet® and DELPHION™ databases were used. DELPHION™ is a private database that provides several information on patents related documents from United States, Japan, Germany or any other patents filed under the European Patent Convention (EPC) or the Patent Cooperation Treaty (PCT). Esp@cenet® is maintained by the European Patents Office.

The following set of key expressions was used: nitrification, ammonia/ammonium/nitrite oxidizer, nitrifying consortia/consortium, Nitrosomonas, Nitrosospira, Nitrospira, Nitrobacter, aquaria, biofilter start-up, Hovanec.

For each search, different types of documents were obtained namely granted patents, utility models, patent applications, search reports and patent translation documents.

Only patents regarding inocula from the same commercial product line as defined in 1.11.1 were considered.

Eight different patent families were found containing a total number of 91 documents including 11 granted patents and 2 utility models. All the granted patents were analysed and 4 criteria (organisms, applications, methods, inoculum form) were noted as relevant to allow direct comparison between patents and evaluation of the state of the art.

2.2.2 Nitrifying inocula (NI)

Four different commercial inocula (ABIL®, Baktinetten®, BioSpira®, FilterStart®) and one product in the test phase (Product A) were studied in terms of their specific nitrifying activity in freshwater.

The nitrifying suspension ABIL® was obtained from Avecom (Belgium). This culture was obtained by gradual enrichment starting from activated sludge. The product contains several autotrophs accompanied by a diverse heterotrophic community and includes CaCO₃ as a carrier matrix. Nitrosomonas aestuarii and Nitrobacter winogradsky can be found as the major nitrifiers in the mixture. For use in domestic aquaria, 27 mL of the product (2.5 g VSS/L) has to be added in a volume of 100 L (6.75×10⁻⁴ g VSS/L).

Baktinetten® was obtained from Söll (Germany). This product is sold in the form of calcium alginate capsules containing an immobilized culture comprising Nitrosomonas sp., Nitrobacter sp. and Paracoccus denitrificans. The commercial dosage to be applied is equal to 50 mL of granules per 100 L of water to be treated.
Biospira® for freshwater, was obtained from Marineland (Aquaria, INC; Spectrum Brands, INC; USA) through a specialized distributor inside the USA (Drs. Foster and Smith, USA). Biospira® is known to comprise Nitrosomonas sp. and Nitrospira sp. in its constitution. According to the manufacturer, a pouch of BioSpira freshwater 3 oz. (88 mL) is able to treat 90 gallons (340 litres) of freshwater.

Filterstart® was obtained from JBL (Germany). The formulation of the product is unknown but according to the advertisement it contains several bacteria responsible by decreasing the levels of TAN, nitrite and nitrate. The used commercial dose is a function of the filter material volume. 10 mL of the product could be applied in a filter up to 3 L.

Product A was obtained from LabMET (Biosciences Faculty, UGhent, Belgium). The nitrifying suspension is immobilized in a calcium alginate matrix in the form of small granules. The product has a secret formulation. A hypothetical commercial dose of 50 mL granules to treat 100 L of water was considered. This dose is identical to the commercial dose of Baktinetten.

### 2.2.3 Specific nitrifying activity of the NI

A series of batch-type tests were carried out in order to determine the specific nitrifying activity of the several NI at TAN concentrations between 5 and 40 mg N-TAN/L and nitrite concentrations between 5 and 20 mg N-NO₂⁻/L. Specific volumes of an ammonium chloride solution (1 g N-TAN/L) were added to 500 mL Erlenmeyer flasks, filled with nitrifying consortia and tap water in order to produce a certain concentration of TAN, nitrite or both.

To assure oxygen availability and a good mixture preventing the cells from settling, each flask was equipped with an air blower.

The tests were set in duplicate and control flasks without biomass, containing tap water, TAN solution and/or NO₂⁻ solution were used. For each test flask, the specific activity was calculated by subtracting the amount of TAN lost in the controls from the TAN removal rate per g VSS, in the test flasks. A variable period of time was used to determine the specific activity.

In order to compare different commercial preparations, a specific activity “per commercial dose” was calculated. Other tests were performed with ABIL and in this case, specific activity “per g VSS” was calculated.

Commercial concentrations of the NI were considered and different concentrations were used for each test. TAN and NO₂⁻ concentration were analyzed daily. NO₃⁻ concentration was analyzed for the first test.

Particular modifications to this general protocol and other specific indications such as pH and temperature are showed below.
2.2.3.1 ABIL (0.05 and 0.025 g VSS/L) at two different TAN concentrations

This test was carried out with TAN and a combination of TAN and nitrite. Concentrations of 20 and 40 mg N-TAN/L and 20 mg N-NO₂⁻/L were used. The temperature of the room varied between 18°C and 22°C. Specific activities were calculated for the first 75 h of test. Table 2.1 summarizes the test conditions. The pH was also measured in the beginning and at the end of the test.

Table 2.1 Summary of the test conditions concerning TAN and NO₂⁻ oxidation by ABIL.

<table>
<thead>
<tr>
<th>Code</th>
<th>TAN concentration (mg N-TAN/L)</th>
<th>NO₂⁻ concentration (mg N-NO₂⁻/L)</th>
<th>Concentration (g VSS/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20A_50</td>
<td>20</td>
<td>20</td>
<td>0.050</td>
</tr>
<tr>
<td>20A_25</td>
<td>20</td>
<td>20</td>
<td>0.025</td>
</tr>
<tr>
<td>20_Control</td>
<td>20</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>40A_50</td>
<td>40</td>
<td>-</td>
<td>0.050</td>
</tr>
<tr>
<td>40A_25</td>
<td>40</td>
<td>-</td>
<td>0.025</td>
</tr>
<tr>
<td>40_Control</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.2.3.2 ABIL and Baktinetten® and a mixture of ABIL and Baktinetten®

Flasks with one type of inoculum were inoculated with an overdosage of 20 times for ABIL and Baktinetten®. As the commercial dosage of Baktinetten® (according to the manufacturer) is about 50 mL for 100 L, 5 mL of granules were placed in the 500 mL flasks. The commercial dosage for ABIL (2.5 g VSS/L) was assumed to be equal to 27 mL for 100 L. Approximately 7 mg VSS of ABIL were placed in each one of the flasks.

An overdosage of ten times for both ABIL and Baktinetten® was used in the flasks with both types of inocula (Mix).

After the first TAN and nitrite pulse in the start of the test, another pulse was given, 124 h after the start of the test.

Two activities were calculated for TAN and nitrite oxidation. The first one was determined considering the first 48 hours of the test and the second, considering the time between t=150h and 175 h after the beginning of the test (after the second pulse).

In order to prevent pH fluctuations, specific volumes of two solutions (0.2 mM KH₂PO₄ and K₂HPO₄) were added to every flask. The average pH in the flasks was tuned to 7.07 (ranging from 7.03 to 7.2) and kept almost constant along the assay.

The temperature of the room varied between 18°C and 25°C. Table 2.2 summarizes the test conditions.
Table 2.2 Summary of the test conditions concerning TAN and NO$_2$ oxidation by ABIL and Baktinetten.

<table>
<thead>
<tr>
<th>Code</th>
<th>TAN concentration (mg N-TAN/L)</th>
<th>ABIL Overdosage</th>
<th>Baktinetten Overdosage</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAN Oxidation</td>
<td></td>
<td></td>
<td></td>
<td>0.2 mM</td>
</tr>
<tr>
<td>ABIL</td>
<td>5</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Baktinetten</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Mix</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Code</th>
<th>NO$_2$ concentration (mg N- NO$_2$/L)</th>
<th>ABIL Overdosage</th>
<th>Baktinetten Overdosage</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite Oxidation</td>
<td></td>
<td></td>
<td></td>
<td>0.2 mM</td>
</tr>
<tr>
<td>ABIL</td>
<td>5</td>
<td>20</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Baktinetten</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Mix</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

2.2.3.3 Biospira$^\text{®}$

According to the manufacturer, a pouch of Biospira$^\text{®}$ freshwater 3 oz. (88 mL) is able to treat 90 gallons (340 litres). Volumes of about 2.5 mL (20 dosages) and 10 mL (80 dosages) were added to the test flasks. The pH in the flasks was kept above 7.5 along the assay. The temperature of the room varied between 25ºC and 30ºC. Table 2.3 summarizes the test conditions. Activities were calculated considering the first 72 h of test.

Table 2.3 Summary of the test conditions concerning TAN and NO$_2$ oxidation by Biospira.

<table>
<thead>
<tr>
<th>Code</th>
<th>TAN concentration (mg N-TAN/L)</th>
<th>Overdosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAN Oxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biospira_20</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Biospira_80</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Code</th>
<th>NO$_2$ concentration (mg N- NO$_2$/L)</th>
<th>Overdosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrite Oxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biospira_20</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Biospira_80</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.2.3.4 Filterstart$^\text{®}$

According to the manufacturer, 10 mL of Filterstart$^\text{®}$ are able to colonize up to 3 L of filter material. Volumes of 2.5 mL were added to the test flasks. Activities were calculated considering the first 72 h of test.

2.2.3.5 Product A
Concerning Product A, a commercial dosage of 50 mL for 100 L of water to treat was considered (the same dose as used for Baktinetten®). 5 and 20 mL of granules were placed in the 500 mL flasks in order to produce overdosages of respectively 20 and 80 times. Duplicates were only used for the 20 times overdosage flasks.

The temperature of the room varied between 25ºC and 30ºC. Table 2.4 summarizes the test conditions. Activities were calculated considering the first 120 h of test.

Table 2.4 Summary of the test conditions concerning TAN and NO$_2^-$ oxidation by Product A.

<table>
<thead>
<tr>
<th>Code</th>
<th>TAN Oxidation</th>
<th>TAN concentration (mg N-TAN/L)</th>
<th>Overdosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProductA_20</td>
<td></td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>ProductA_80</td>
<td></td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Code</th>
<th>Nitrite Oxidation</th>
<th>NO$_2^-$ concentration (mg N-NO$_2^-$/L)</th>
<th>Overdosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProductA_20</td>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>ProductA_80</td>
<td></td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

2.2.3.6 Influence of phosphorous in the nitrifying activity of ABIL

The influence of adding phosphorous (as KH$_2$PO$_4$) to the test flasks was tested only for ABIL. Two different ratios N/P were used. TAN concentration was always equal to 5 mg N-TAN/L. In every test flask, an inoculum concentration of 1.0 mg VSS/L was used (approximately 1.5 times the commercial dosage).

Table 2.5 Summary of the test conditions concerning TAN oxidation by ABIL with extra P addition.

<table>
<thead>
<tr>
<th>Code</th>
<th>TAN concentration (mg N-TAN/L)</th>
<th>P concentration (mg P/L)</th>
<th>Amount of inoculum (mg VSS/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No P</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>N/P = 5</td>
<td>5</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>N/P = 2.5</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

2.2.3.7 Influence of adding several TAN pulses

The influence of the re-activation of the stored commercial product ABIL by adding several subsequent TAN pulses was tested. TAN starting concentration was equal to 5 mg N-TAN/L. Two more pulses were added at t=73 and 144 h. At the start, a volume equal to 2.7 mL of inoculum was added in order to produce concentrations of 6.75 mg VSS/L (10 times the commercial dosage).

2.2.4 Biolog test
The affinity of ABIL and aerobic activated sludge (AAS) for different carbon sources (CS) was evaluated using microplates from Biolog (Biolog Inc., USA). The activated sludge was used as an example of a diverse heterotrophic community. The sample was collected in a sewage treatment plant (WWTP) (Ossemeersen WWTP, Ghent, Belgium).

Eight Biolog GN 2 microtiter plates were inoculated with either ABIL or AAS in four different dilutions (from $2.5 \times 10^{-1}$ to $2.5 \times 10^{-4}$ g VSS/L) and incubated at 28ºC.

The optical density at 590 nm generated from the reduction of tetrazolium violet in each well was read after 24 and 72 hours using a Tecan (USA) microplate reader. Before further processing, the OD$_{590}$ for the control well was subtracted from the later readings for each C-source.

Six hours after inoculation no colour development was noticed in any of the wells (data not shown).

For each one of the eight plates, 24 and 72 hours after inoculation, CS with stronger signals (equal or bigger than the average plus 2×Standard Deviation) were considered.

From this data set all the CS with an occurrence frequency ≥ 2 were registered in order to find the most commonly oxidized CS for ABIL and AAS.

The average signal in each one of the plates was also calculated in order to compare the relative oxidation versatility and activity for Activated Sludge and ABIL.

### 2.2.5 Chemical oxygen demand (COD) tests

In order to study the oxidation rate of different CS by ABIL and AAS, several test flasks were inoculated with ABIL and AAS. A certain amount of each carbon source was weighted and added to each flask. No buffer was added but the pH was always kept at values in the range 7.4 – 8.3. The COD$_{\text{soluble}}$ content for each flask was measured as a function of time.

The temperature in the room was kept between 24-28 ºC and the flasks were placed in a shaker at 125 RPM.

Two specific volumes of NH$_4$Cl and KH$_2$PO$_4$ solutions were added in order to generate a ratio COD/N/P equal to 100/5/1.

Samples were collected in different moments and filtrated with 0.45 µm syringe filters.

Particular modifications to this general protocol and other specific indications are showed below.

#### 2.2.5.1 Oxidation activity of ABIL and two types of aerobic activated sludge for different carbon sources

Seven 1-L erlenmeyer flasks with different CS plus one control without CS were inoculated with ABIL at a concentration of about 0.1 g VSS/L. Three hours before starting the test, chill stored ABIL at 4ºC was brought to the room temperature. Samples were collected at t=0, 2, 24 and 48 h.

For each AAS sample, 5 flasks plus one control without CS were inoculated with AAS producing concentrations of about 0.1 g VSS/L.
Samples of activated sludge, type 1 (2 g VSS/L) and type 2 (6 g VSS/L) were collected respectively in a sewage treatment plant (WWTP) (Ossemeersen WWTP, Ghent, Belgium) and in an aerobic WWTP of a Slaughterhouse (Cominbel NV, Sint-Lievens-Houtem, Belgium) and the tests were performed less than 24 h after collecting these samples. During this test series, samples were collected at t=0, 3, 6 and 21 h.

Table 2.6 summarizes starting conditions for each one of the flasks inoculated with ABIL and AAS.

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>COD content (g COD/g CS)</th>
<th>Initial COD Concentration (mg/L) ABIL</th>
<th>Initial COD Concentration (mg/L) AAS type 1</th>
<th>Initial COD Concentration (mg/L) AAS type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.07</td>
<td>191</td>
<td>290</td>
<td>226</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.12</td>
<td>160</td>
<td>190</td>
<td>252</td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td>1*</td>
<td>135</td>
<td>138</td>
<td>179</td>
</tr>
<tr>
<td>Peptone</td>
<td>1*</td>
<td>363</td>
<td>317</td>
<td>336</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>0.98</td>
<td>-</td>
<td>193</td>
<td>170</td>
</tr>
<tr>
<td>Tween 80</td>
<td>2</td>
<td>388</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calcium Acetate</td>
<td>0.71</td>
<td>160</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>1.12</td>
<td>180</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(1) arbitrary values not calculated

2.2.5.2 Oxidation activity of ABIL and two types of aerobic activated sludge for peptone

In order to study the oxidation rate of peptone, 3 duplicates plus 3 control flasks (without peptone) were inoculated with ABIL and AAS (type 1 and 2) at a concentration of 250 mg VSS/L. Samples of activated sludge were used less than 36 h after being collected. Thirty hours before starting the test, chill stored ABIL at 4°C was brought to the room temperature and fed with a total amount of 50 mg N-TAN/g VSS and 50 mg N-NO₂/g VSS. A specific amount of peptone was added in order to produce concentrations of about 400 mg COD/L. Samples were collected at t=0, 1, 2, 3, 4 and 6 h.

2.2.5.3 Oxidation activity of ABIL and aerobic activated sludge (type 2) for glucose

In order to study the oxidation rate of glucose, the oxidation rate of glucose was measured immediately after one overnight starvation period of the inoculum (ABIL or AAS) without adding any type of feeding. The adsorption capacity of both Activated Sludge and ABIL was also studied by measuring the CODsoluble content variation as a function of time for autoclaved Activated Sludge and ABIL.

Ten Erlenmeyer flasks (2 test flasks + 2 autoclaved controls + 1 control without glucose addition for each one of the inocula) were inoculated with ABIL and AAS at a concentration of respectively 2.5 and 2.9 g VSS/L.
A specific amount of glucose was added in order to produce concentrations of about 1250 mg/L generating a loading ratio of about 0.4 g COD/g VSS. Samples were collected at t=0, 1, 2, 3 and 4 h.

2.2.6 Oxygen uptake rate (OUR) tests

The OUR tests were performed by measuring the dissolved oxygen as a function of time. The tests were performed in order to compare ABIL and two types of activated sludge (mixed liquor) in terms of their specific OUR. Several authors have estimated kinetic parameters by obtaining respirometric measurements. In these types of tests, concentration of oxygen in solution will decrease due to substrate consumption (Ginestet et al, 1998). By plotting the measured DO values against time it is possible to determine directly the OUR of a certain inoculum for a specific substrate.

Samples of activated sludge, type 2 (3.2 g VSS/L) and type 3 (2.9 g VSS/L) were collected respectively in a slaughterhouse (Cominbel NV, Sint-Lievens-Houtem, Belgium) and in a paper mill (Stora Enso’s Langerbrugge Mill, Ghent, Belgium).

Samples of activated sludge (type 2 and type 3) were obtained. The samples 2 and 3 presented an approximate VSS content of respectively 3.2 g VSS/L and 2.9 g VSS/L.

The flask was equipped with a DO electrode and a sparge connected to an air pump (5 L air/min).

By air blowing it was possible to attain oxygen saturation. In order to determine endogenous OUR, after attaining oxygen saturation (that could be noticed by the DO stabilization), the air pump was switched off and the DO was measured as a function of time.

In order to determine OUR as a consequence of addition of the oxidized compound, after air blowing the air pump was switched off and 10 mL of a solution of glucose or NH₄Cl was added (generating concentrations of about 100 mg glucose/L or 100 mg N-TAN/L).

The DO is given in mg/L, the OUR_{endo} was calculated according to equation 18:

\[
\text{OUR}_{\text{endo}} = \frac{\text{DO}_i - \text{DO}_t}{t} \times \frac{1}{\text{VSS}_{\text{content}}}
\]

DO_i and DO_t represent respectively DO at saturation and DO after a time t without air blowing.

The OUR_{glucose} and OUR_{TAN} were calculated in the same way after subtraction of OUR_{endo} value. These values as well as the ratio OUR_{glucose}/OUR_{TAN} were determined.

All the tests were performed between 28 ºC and 32 ºC (temperature in the liquid) always with gentle stirring (without vortex formation). pH was kept at about 7.5 for ABIL and sludge B and above 7 for sludge A.
2.2.7 Analytical methods

2.2.7.1 TAN concentration by using a colorimetric method

TAN concentration was determined by using the direct photometric method with Nessler’s reagent (Greenberg et al, 1992). In the first step of the method, sodium potassium tartrate is added in order to capture interfering elements such as Mn and Fe. Afterwards Nessler’s reagent is added generating a yellow alkaline solution by complexing ammonia with HgI$_4^{2-}$ ions present in the Nessler’s reagent. Intensity of the yellow colour is proportional to the concentration of TAN in the sample. The absorbance is measured at 425 nm using a UVIKON 932 spectrophotometer (Kontron instruments, Switzerland).

2.2.7.2 Nitrite concentration

2.2.7.2.1 Ion-exchange (IC) chromatography

Concerning the first specific nitrifying activity test, nitrite concentration was measured by using a 761 Compact Ion Cromatograph (Metrohm, Switzerland) equipped with an anionic column (Metrosep A Supp 5 IC) preceded by a guard column (Metrosep A Supp 4/5 Guard).

2.2.7.2.2 Colorimetric method

Nitrite concentration was determined for most of the samples by using a direct photometric method according to Montgomery & Dymock (1961).

In the first step of the method, sulphanilic acid solution is added. Afterwards a solution of N-(1-naphthyl) ethylenediamine hydrochloride is added. The absorbance is measured at 550 nm using a UVIKON 932 spectrophotometer (Kontron instruments, Switzerland).

2.2.7.3 Nitrate concentration

2.2.7.3.1 Ion-exchange (IC) chromatography

Nitrate concentration was always determined by using an IC apparatus as performed for nitrite determination.

2.2.7.4 TSS & VSS

The volatile suspended solids content of Activated Sludge was determined by subtracting the ash content from the total suspended solids (TSS) content. Ash content and TSS content were determined gravimetrically (Greenberg et al, 1992).

2.2.7.4.1 Chemical oxygen demand (COD)

The COD content was determined by measuring the excess of a solution of potassium dichromate after complete oxidation of the sample in acidic conditions (Greenberg et al, 1992).
2.2.7.5 Dissolved oxygen (DO) and pH

The pH and DO were measured by using respectively a C 532 digital pH-meter (Consort, Belgium) and a COM 280 DO meter (Endress + Hauser, Germany).

2.3 Results

2.3.1 Patents survey

A total number of 11 granted patents and two utility models were found in six different countries (USA (4), Germany (3), Australia (2), Mexico (2), Austria (1), and Belgium (1)). The patents were filed by four different companies (Aquaria, INC; Co-operative Research Centre (CRC) for Waste Management and Pollution Control Limited (WMPC), Avecom NV and Söll GmbH).

Aquaria, INC and its brands belong to Spectrum Brands, INC, an American Company that also owns Tetra Holdings GmbH, United Pet Group, Inc. and Jungle Laboratories Corporation (together, Global Pet) (Reuters Corporate, 2007).

The CRC-WMPC was established and supported under the Australian Governments Cooperative Research Centres Program and became an Incorporated Company in July 1992 (CRC, 2007). Söll GmbH is a German company that offers a wide range of water treatment products.

Avecom N.V. is a Belgian company that works in close collaboration with The University of Ghent, of which it’s a spin-off (Avecom, 2007). From these three companies Avecom N.V., Aquaria, Inc and Söll GmbH are known to produce nitrifying inocula. ABIL® (ammonium binding inoculum liquid) was developed to be applied in aquaculture systems and aquaria/ponds and is produced by Avecom. BioSpira® and Baktinetten® are produced respectively by Aquaria, Inc and Söll GmbH for domestic purposes (aquaria/ornamental ponds).

11 out of 13 collected patents have direct relationships with the producers of the studied inocula. Tables 2.7 and 2.8 make a summary on general aspects of the selected patents. In order to compare the collected patents in terms of their most important claims, four criteria were considered as relevant. Table 2.9 presents a summary of these criteria as well as which ones of the criteria are effectively claimed.
Table 2.7 Summary on general aspects of the granted patents selected after the survey: applicant (company), commercial denomination (product), countries where the patents were granted.

<table>
<thead>
<tr>
<th>Patents / Utility Models</th>
<th>Company</th>
<th>Product</th>
<th>Countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>BE 1012344</td>
<td>Avecom</td>
<td>ABIL</td>
<td>Belgium (1)</td>
</tr>
<tr>
<td>US 6265206</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 6268154</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>US 6207440</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DE 60109441</td>
<td>Marineland</td>
<td>BioSpira®</td>
<td>USA (3); Germany (1); Australia (1); Austria (1); Mexico (2)</td>
</tr>
<tr>
<td>AU 0750945</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT 0291078</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MX 5002873</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MX 2011412</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DE 20207625 U1</td>
<td>Söll</td>
<td>Baktinetten®</td>
<td>Germany (2)</td>
</tr>
<tr>
<td>DE 20 2004 007620 U1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.8 Summary on the main aspects used in order to compare patents.

<table>
<thead>
<tr>
<th>Number</th>
<th>Patent Title</th>
<th>Publication Date</th>
<th>Patent Nº</th>
<th>Patent Family (Total number of documents in the family)</th>
<th>Applicant</th>
<th>Country</th>
<th>Inventor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Method of using bacterial nitrite oxidizer</td>
<td>24/07/2001</td>
<td>US 6265206</td>
<td>A (11)</td>
<td>Aquaria, INC</td>
<td>USA</td>
<td>Hovanec, Timothy A.</td>
</tr>
<tr>
<td>2</td>
<td>Method for detecting bacterial nitrite oxidizer</td>
<td>31/07/2001</td>
<td>US 6268154</td>
<td>A (11)</td>
<td>Aquaria, INC</td>
<td>USA</td>
<td>Hovanec, Timothy A.</td>
</tr>
<tr>
<td>3</td>
<td>Bacterial nitrite oxidizer</td>
<td>27/03/2001</td>
<td>US 6207440</td>
<td>A (11)</td>
<td>Aquaria, INC</td>
<td>USA</td>
<td>Hovanec, Timothy A.</td>
</tr>
<tr>
<td>4</td>
<td>Method for the detection of aquatic nitrite oxidizing microorganisms of the genus Nitrospira</td>
<td>24/04/2001</td>
<td>US 6221594</td>
<td>B (6)</td>
<td>CRC for Waste Management and Pollution Control Limited</td>
<td>USA</td>
<td>Burrell, Paul Christopher; Blackall, Linda Louise; Keller, Jurg</td>
</tr>
<tr>
<td>5</td>
<td>Ammoniak-Oxidierende Bakterien</td>
<td>21/04/2005</td>
<td>DE 60109441</td>
<td>C (26); E (18)</td>
<td>Aquaria, INC</td>
<td>Germany</td>
<td>Burrell, Paul Christopher; Hovanec, Timothy A.</td>
</tr>
<tr>
<td>6</td>
<td>Aquatic nitrite oxidizing microorganisms</td>
<td>10/10/2002</td>
<td>AU 0753130</td>
<td>B (6)</td>
<td>CRC for Waste Management and Pollution Control Limited</td>
<td>Australia</td>
<td>Burrell, Paul C; Blackall, Linda L; Keller, Jurg</td>
</tr>
<tr>
<td>7</td>
<td>Bacterial nitrite oxidizer and method of use thereof</td>
<td>01/08/2002</td>
<td>AU 0750945</td>
<td>A (11)</td>
<td>Aquaria, INC</td>
<td>Australia</td>
<td>Hovanec, Timothy A.</td>
</tr>
<tr>
<td>8</td>
<td>Ammonia oxidizing bacteria and methods of using and detecting the same</td>
<td>22/06/2005</td>
<td>MX 5002873</td>
<td>F (11)</td>
<td>Aquaria, INC</td>
<td>Mexico</td>
<td>Hovanec, Timothy A.</td>
</tr>
<tr>
<td>9</td>
<td>Ammonia Oxidizing Bacteria</td>
<td>26/02/2004</td>
<td>MX 2011412</td>
<td>E (18)</td>
<td>Aquaria, INC</td>
<td>Mexico</td>
<td>Hovanec, Timothy A.</td>
</tr>
<tr>
<td>10</td>
<td>Ammoniak-Oxidierende Bakterien</td>
<td>15/04/2005</td>
<td>AT 0291078</td>
<td>E (18)</td>
<td>Aquaria, INC</td>
<td>Austria</td>
<td>Burrell, Paul Christopher; Hovanec, Timothy A.</td>
</tr>
<tr>
<td>11</td>
<td>A supplementary product for the removal of ammonia</td>
<td>29/05/1998</td>
<td>BE 1012344</td>
<td>G (2)</td>
<td>Avecom N.V.</td>
<td>Belgium</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Formkörper, enthaltend mikrobiologische Kulturen</td>
<td>13/11/2003</td>
<td>DE 20207625 (Type U1 – Utility Model)</td>
<td>None</td>
<td>Söll GmbH</td>
<td>Germany</td>
<td>Christophersen, R.</td>
</tr>
<tr>
<td>13</td>
<td>Aufbewahrungs- und Dosiervormcitung für Mikroorganismen</td>
<td>27/10/2005</td>
<td>DE 20 2004 007620 (Type U1 – Utility Model)</td>
<td>None</td>
<td>Söll GmbH</td>
<td>Germany</td>
<td>-</td>
</tr>
<tr>
<td>Number</td>
<td>Organisms</td>
<td>Applications</td>
<td>Method</td>
<td>Form</td>
<td>Claims include</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>--------------</td>
<td>--------</td>
<td>------</td>
<td>----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>NOB's having at least 96.1% similarity to a certain 16 S rDNA sequence belonging to a <em>Nitrospira moscowiensis</em> related organism</td>
<td>Bioremediation process that alleviates or prevents accumulation of nitrite in saltwater/freshwater aquaria or wastewater by using the specified organism</td>
<td>The specified organism is brought into the medium by placing it in rotating biological contactors or biofilters</td>
<td>-</td>
<td>Organism/Compositions comprising the organism, specified applications and methodology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>NOB's having 100% similarity to a certain 16 S rDNA sequence belonging to a certain organism from the <em>Nitrospira</em> genus</td>
<td>Method for detecting and determining the amount of the given NOB contained in aquaria or wastewater</td>
<td>The detection of the specified organism is made by providing a detectably labeled probe and isolating total DNA from the media</td>
<td>-</td>
<td>Specified applications and method</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NOB's having at least 96.1% similarity to a certain 16 S rDNA sequence belonging to a certain organism from the <em>Nitrospira</em> genus. Compositions including the nitrifying species and other organisms (AOB’s, nitrate-reducing, heterotrophic or combinations) are also claimed</td>
<td>Method for detecting and determining the amount of the specified NOB in a sample</td>
<td>The detection is made by providing a detectably labeled probe and isolating total DNA from the media</td>
<td>frozen, freeze-dried or powdered</td>
<td>Organism/Compositions comprising the organism and form</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NOB's belonging to the <em>Nitrospira</em> genus</td>
<td>Method for detecting and determining the amount of a certain NOB in a sample</td>
<td>The detection is made by providing a detectably labeled probe and isolating total DNA from the media</td>
<td>-</td>
<td>Specified applications and method</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>AOB’s having at least 99% similarity to a certain 16 S rDNA sequence belonging to a certain organism from the genus <em>Nitrosospira</em> or <em>Nitrosomonas</em>. Compositions including the nitrifying species and other organisms (AOB’s, nitrate-reducing, heterotrophic or combinations) are also claimed</td>
<td>Bioremediation process that alleviates or prevents accumulation of ammonia in aquaria</td>
<td>The specified organism is brought into the medium by placing it in rotating biological contactors or biofilters</td>
<td>frozen, freeze-dried or powdered</td>
<td>Organism/Compositions comprising the organism, specified applications, methodology and form</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>NOB’s belonging to the <em>Nitrospira</em> genus</td>
<td>Method for detecting and determining the amount of the specified NOB in a sample</td>
<td>The detection is made by providing a detectably labeled probe and isolating total DNA from the media</td>
<td>-</td>
<td>Specified applications and method</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>NOB's having at least 96.1% similarity to a certain 16 S rDNA sequence belonging to a certain organism from the genus <em>Nitrospira</em> or <em>Nitrosomonas</em>. Compositions including the nitrifying species and other organisms (AOB’s, nitrate-reducing, heterotrophic or combinations) are also claimed</td>
<td>Bioremediation process that alleviates or prevents accumulation of nitrite in saltwater/freshwater aquaria or wastewater by using the specified organism</td>
<td>The specified organism is brought into the medium by placing it in rotating biological contactors or biofilters</td>
<td>frozen, freeze-dried or powdered</td>
<td>Organism/Compositions comprising the organism, specified applications, methodology and form</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AOB’s having at least 99% similarity to a certain 16 S rDNA sequence belonging to a certain organism from the <em>Nitrospira</em> genus Compositions including the nitrifying species and other organisms (AOB’s, nitrate-reducing, heterotrophic or combinations) are also claimed</td>
<td>Bioremediation process that alleviates or prevents accumulation of ammonia in aquaria</td>
<td>The specified organism is brought into the medium by placing it in rotating biological contactors or biofilters</td>
<td>frozen, freeze-dried or powdered</td>
<td>Organism/Compositions comprising the organism, specified applications, methodology and form</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>AOB’s having a certain similarity to a given 16 S rDNA sequence belonging to an AOB</td>
<td>Bioremediation process that alleviates or prevents accumulation of ammonia in aquaria</td>
<td>The specified organism is brought into the medium by placing it in rotating biological contactors or biofilters</td>
<td>frozen, freeze-dried or powdered</td>
<td>Organism/Compositions comprising the organism, specified applications, methodology and form</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>AOB’s having at least 99% similarity to a certain 16 S rDNA sequence belonging to a certain organism from the genus <em>Nitrospira</em> or <em>Nitrosomonas</em> genus. Compositions including the nitrifying species and other organisms (AOB’s, nitrate-reducing, heterotrophic or combinations) are also claimed</td>
<td>Bioremediation process that alleviates or prevents accumulation of ammonia in aquaria</td>
<td>The specified organism is brought into the medium by placing it in rotating biological contactors or biofilters</td>
<td>frozen, freeze-dried or powdered</td>
<td>Organism/Compositions comprising the organism, specified applications, methodology and form</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Highly active inoculum of nitrifiers formulated with CaCO₃, as a carrier and growth matrix.</td>
<td>Bioremediation process that alleviates or prevents accumulation of ammonia, nitrite and nitrate in aquaria</td>
<td>Direct appliance in aquatic environments Used carrier improves settling times allowing collection and enrichment of the culture</td>
<td>liquid</td>
<td>Composition comprising the organisms, specified applications, methodology and form</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Heterotrophic nitrifiers and denitifiers</td>
<td>Process to start micro-biological processes in water</td>
<td>The inoculum is delivered as a molded article manufactured over injecting procedures</td>
<td>Molded matrix containing freeze dried inoculum</td>
<td>Composition comprising the organisms, specified applications, methodology and form</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Method to store the nitrifying inoculum providing aeration and feeding with a device that allows distribution of determined amounts of the same</td>
<td>The storage unit keeps the inoculum in aqueous solution and has an air intake The metering unit is coupled with the storage and allows proportioned delivery of inoculum</td>
<td>-</td>
<td>Application and method</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3.2 Specific nitrifying activity of the NI

A series of batch-type tests were carried out in order to determine the specific nitrifying activity of the different commercial products. The influence of several consecutive additions of TAN and the influence of phosphorous addition was also tested for ABIL. Apart from 3.2.7, in all the tests specific nitrifying activity was calculated by subtracting the amount of TAN lost in a control from the TAN removal rate in the tests with TAN and/or nitrite addition.

2.3.2.1 ABIL (0.050 and 0.025 g VSS/L) at two different TAN concentrations

In this test, two concentrations of ABIL (0.050 and 0.025 g VSS/L) were placed in the erlenmeyer flasks and tested in the presence of two different TAN pulses of 20 and 40 mg N-TAN/L. An amount of 20 mg of N-NO$_2$-L was also added only for the flasks starting with the initial 20 mg N-TAN/L pulse (Figures 2.1 and 2.2).

![Figure 2.1](image1.png)

**Figure 2.1** Concentrations of TAN, N-NO$_2$ and N-NO$_3$ as a function of time, following the addition of one pulse of 20 mg N-TAN/L and one pulse of 20 mg N-NO$_2$L, to the 500 mL test erlenmeyers inoculated with 0.025 g VSS/L ABIL. Curves represent the average values obtained with the duplicates. Standard deviations are represented by the bars.

![Figure 2.2](image2.png)

**Figure 2.2** Concentrations of TAN, N-NO$_2$ and N-NO$_3$ as a function of time, following the addition of one pulse of 20 mg N-TAN/L to the 500 mL test erlenmeyers inoculated with 0.050 g VSS/L ABIL. Curves represent the average values obtained with the duplicates. Standard deviations are represented by the bars.
In the four different situations considered in this test series (2 ABIL concentrations × 2 different TAN pulses) the obtained specific activities were quite similar ranging from 0.086 to 0.201 g N-TAN /g VSS.d. The average activity value was found to be equal to 0.134±0.032 g N-TAN /g VSS.d. During the test it was possible to notice a slight decrease on the pH in every flask but the latter was always kept above 7.3. Table 2.10 makes a summary on all the activities measured over the course of this test.

Table 2.10 Activities obtained for each one of the tested flasks, concerning the test series with ABIL.

<table>
<thead>
<tr>
<th>Code</th>
<th>TAN oxidation activity (g N-TAN /g VSS d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20A25_1</td>
<td>0.086</td>
</tr>
<tr>
<td>20A25_2</td>
<td>0.130</td>
</tr>
<tr>
<td>20A50_1</td>
<td>0.115</td>
</tr>
<tr>
<td>20A50_2</td>
<td>0.153</td>
</tr>
<tr>
<td>40A25_1</td>
<td>0.114</td>
</tr>
<tr>
<td>40A25_2</td>
<td>0.134</td>
</tr>
<tr>
<td>40A50_1</td>
<td>0.201</td>
</tr>
<tr>
<td>40A50_2</td>
<td>0.138</td>
</tr>
<tr>
<td>Average</td>
<td>0.134 ± 0.032</td>
</tr>
</tbody>
</table>

2.3.2.2 ABIL and Baktinetten® and a mixture of ABIL and Baktinetten®

Concerning this test series, there was no combined addition of TAN and NO₂⁻. The latter were added separately in order to determine two specific activities for TAN and NO₂⁻ oxidation. The used inoculums’ overdosage was equal to 20 when using the pure commercial products and 10 when the two tested inocula were combined (Figures 2.3 and 2.4).

Figure 2.3 Concentrations of TAN as a function of time, following the addition of one pulse of approximately 5 mg N-TAN/L to the 500 mL flasks. A second pulse was added 124 h after the test start. Curves represent the average values obtained with the duplicates for each one of the inocula (ABIL, Baktinetten and a mixture of ABIL and Baktinetten). Standard deviations are represented by the bars.
Figure 2.4 Concentrations of N-NO$_2$ as a function of time, following the addition of one pulse of approximately 5 mg N-NO$_2$/L to the 500 mL flasks. A second pulse was added 124 h after the test start. Curves represent the average values obtained with the duplicates for each one of the inocula (ABIL, Baktinetten and a mixture of ABIL and Baktinetten). Standard deviations are represented by the bars.

Table 2.11 makes a summary on all the activities measured over the course of this test series. Activities for each one of the periods of time were considered according to 2.2.3. Apart from the commercial specific activity, the specific activity of ABIL in terms of the VSS content was also determined for both periods of time. Along the test, the pH was kept above 7 for all the flasks.

Table 2.11 Average activities obtained for each one of the duplicates, concerning the test series with ABIL and Baktinetten.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Activity 1</th>
<th>Activity 2</th>
<th>Activity 1</th>
<th>Activity 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAN</td>
<td>g N-TAN/g VSS.d</td>
<td>g N-TAN/dosage.d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABIL</td>
<td>0.249±0.011</td>
<td>0.320±0.025</td>
<td>0.084±0.01</td>
<td>0.108±0.008</td>
</tr>
<tr>
<td>Baktinetten</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0.011±0.016</td>
</tr>
<tr>
<td>MIX</td>
<td>0.014±0.01</td>
<td>0.0745±0.026</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>g N-TAN/dosage.d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABIL</td>
<td>0.313±0.019</td>
<td>0.103±0.052</td>
<td>0.1055±0</td>
<td>0.0325±0.016</td>
</tr>
<tr>
<td>Baktinetten</td>
<td>-</td>
<td>-</td>
<td>0.002±0</td>
<td>0.0025±0.004</td>
</tr>
<tr>
<td>MIX</td>
<td>0.113±0.01</td>
<td>0.005±0.007</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3.2.3 Biospira®

All over the course of this test, relatively high temperatures were registered. Even though the pH was kept close to 7.5, the temperature in the room reached 30°C during the test, what could explain the decrease in terms of TAN concentration observed in the control flask. A higher amount of stripped ammonia given the higher temperature, could be responsible for the TAN concentration decrease.

Observed TAN oxidation specific activities were equal to zero or, in the case of an overdosage of 80 times, about 2 mg N-TAN/dosage.d. No second pulse of TAN or nitrite was added since the extremely low activity noticed after 72 h of test (data not shown).
Figure 2.5 Concentrations of TAN as a function of time, following the addition of one pulse of approximately 5.0 mg N-TAN/L to the 500 mL flasks and after addition of Biospira. Curves represent the average values obtained with the duplicates. Standard deviations are represented by the bars.

2.3.2.4 Filterstart®

This test was performed for 72 h, after inoculation of the test flasks with a volume of 2.5 mL of Filterstart®. As previously seen in 2.2.5, 10 mL of the inoculum should be able to colonize up to 3 L of filter material. The amount of Filterstart® would thus be able to colonize up to 650 mL of filter material. During the whole test period (72 h) it was not possible to notice significant activities. All the test flasks had slightly lower removals than the control. The pH in the flasks was kept above 7.5 along the assay. The temperature of the room varied between 25ºC and 30ºC.

2.3.2.5 Product A

Product A was dosed to the test flasks in a similar way as performed in 3.2.2. A hypothetical commercial dose of 50 mL for 100 L of nitrogen loaded water was considered. The added TAN pulses, produced concentrations ranging from 6 to 8 mg N-TAN/L (instead of the expected 5 mg N-TAN/L) probably due to uncertainties while preparing the stock solutions and feeding the test flasks. Activities were calculated considering the first 120 h after the pulses. Average TAN and NO$_2^-$ oxidation activities were found to be respectively 0.020±0.012 mg N-TAN/dosage and 0.030±0.010 mg N-NO$_2^-$/dosage. Figures 2.6 and 2.7 represent the obtained profiles for TAN and N-NO$_2^-$ as a function of time for each one of the flasks, since duplicates were only used for the 20 times overdosage flasks.
2.3.2.6 Influence of phosphorous on the nitrifying activity of ABIL

A hypothetical influence of phosphorous on the TAN oxidation activity was only verified for ABIL. Apart from one control without P addition, 2 different ratios N/P were tested. The inoculum was always added at 1.0 mg VSS/L. Activities were calculated considering the first 116 h after the addition of the TAN pulse of about 5 mg N-TAN/L. Average activity for ABIL in the control flasks (no P addition) was found to be equal to 0.296±0.037. As expected, this activity increased more than two times after adding P at two different ratios. The obtained activity values were 0.484±0.112 and 0.644±0.323 g N-TAN/g.VSS d, respectively for the ratio N/P=2.5 and the ratio N/P=5.
2.3.2.7 Influence of adding several TAN pulses

The possibility of stimulating the re-activation of the stored liquid ABIL by adding several TAN pulses to the test flasks was tested. The inoculum was added in order to produce concentrations of approximately 6.75 mg VSS/L.

Activities were calculated for the entire period in between the TAN pulses. After the first pulse, it was possible to observe total oxidation of ammonia. The second pulse was added when a remaining TAN concentration of more than 2 mg N-TAN/L was present in the flasks, generating concentrations of up to 14 mg N-TAN/L. After the first pulse, specific activity was in the range of 0.256±0.016 g N-TAN/g VSS.d. Specific activities after the second and the third pulses were found to be respectively 0.454±0.024 and 0.582±0.093 TAN/g VSS.d (Figures 2.8). The last one was almost two times the measured activity after the first pulse.

![Figure 2.8 Concentrations of TAN as a function of time, following the addition of the three TAN pulses. Curves represent the exact values obtained with each one of the test flasks.](image)

2.3.3 Biolog test

Eight Biolog GN 2 microplates were inoculated in order to identify the ability of carbon oxidation by ABIL and AAS. The inoculations with AAS were used as a representative sample of a diverse heterotrophic community that should present high affinity for some CS and a generalized ability to degrade a high number of CS.

Six hours after inoculation no color development was noticed in any of the wells of the eight plates. Therefore, absorbance of all the plates was measured 24 and 72 hours after inoculation. The average signal for each plate was determined by subtracting the control signal from the measured signal. Table 2.12 presents this average signal for each plate. This value can be considered as a rough estimation of the degree of affinity of both inocula for a given set of 95 CS’s.
Table 2.12 Results of the Biolog tests with ABIL or AAS as inoculum. Average signals for each plate and each inoculum are presented at four different dilutions.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Time (h)</th>
<th>2.5×10⁻⁴</th>
<th>2.5×10⁻³</th>
<th>2.5×10⁻²</th>
<th>2.5×10⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABIL</strong></td>
<td>24</td>
<td>0.033</td>
<td>0.021</td>
<td>0.074</td>
<td>0.319</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.054</td>
<td>0.289</td>
<td>0.540</td>
<td>0.750</td>
</tr>
<tr>
<td><strong>AAS</strong></td>
<td>24</td>
<td>0.018</td>
<td>0.054</td>
<td>0.029</td>
<td>0.502</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.110</td>
<td>0.320</td>
<td>0.692</td>
<td>0.919</td>
</tr>
</tbody>
</table>

CS’s with stronger signals were considered according to the methodology described in 2.2.4 and assigned to a data set. From this data set, all the CS’s with an occurrence frequency ≥ 2 were registered (Table 2.13).

This methodology was performed in order to estimate what could be the most commonly oxidized CS’s by ABIL and AAS.

Table 2.13 Most frequently oxidized CS’s by AAS and ABIL in the Biolog tests. Coloured cells represent CS that were commonly oxidized by both inocula.

<table>
<thead>
<tr>
<th>Sugar Code</th>
<th>Freq.</th>
<th>Sugar Code</th>
<th>Freq.</th>
<th>Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>C7</td>
<td>5</td>
<td>ABIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D12</td>
<td>5</td>
<td>γ-Hydroxybutyric Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>4</td>
<td>Citric Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10</td>
<td>3</td>
<td>Maltose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B11</td>
<td>3</td>
<td>D-Mannitol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td>3</td>
<td>D-Trehalose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>3</td>
<td>cis-Aconitic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G8</td>
<td>3</td>
<td>D-Serine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>3</td>
<td>Uridine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A11</td>
<td>2</td>
<td>D-Arabitol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A12</td>
<td>2</td>
<td>D-Cellobiose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>2</td>
<td>Tween 40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A6</td>
<td>2</td>
<td>Tween 80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B12</td>
<td>2</td>
<td>D-Mannose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7</td>
<td>2</td>
<td>m-Inositol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C11</td>
<td>2</td>
<td>Pyruvic Acid Methyl Ester</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D11</td>
<td>2</td>
<td>β-Hydroxybutyric Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7</td>
<td>2</td>
<td>D-Gluconic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>2</td>
<td>α-Keto Glutaric Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F10</td>
<td>2</td>
<td>L-Glutamic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>2</td>
<td>Glucuronamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>2</td>
<td>Hydroxy-1-Proline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G5</td>
<td>2</td>
<td>L-Phenylalanine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H10</td>
<td>2</td>
<td>D,L-α-Glycerol Phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H11</td>
<td>2</td>
<td>α-D-Glucose-1-Phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9</td>
<td>2</td>
<td>Glycerol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sugar Code</th>
<th>Freq.</th>
<th>Sugar Code</th>
<th>Freq.</th>
<th>Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6</td>
<td>5</td>
<td>Tween 80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>5</td>
<td>D-Fructose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C11</td>
<td>5</td>
<td>Pyruvic Acid Methyl Ester</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td>5</td>
<td>D-Trehalose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A7</td>
<td>4</td>
<td>N-Acetyl-D-Galactosamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B11</td>
<td>4</td>
<td>D-Mannitol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D5</td>
<td>4</td>
<td>D-Galactonic Acid Lactone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B12</td>
<td>3</td>
<td>D-Mannose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td>3</td>
<td>D-Sorbitol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C7</td>
<td>3</td>
<td>Sucrose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C9</td>
<td>3</td>
<td>Turanose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G8</td>
<td>3</td>
<td>D-Serine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>3</td>
<td>Uridine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9</td>
<td>3</td>
<td>Glycerol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A8</td>
<td>2</td>
<td>N-Acetyl-D-Glucosamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10</td>
<td>2</td>
<td>Maltose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>2</td>
<td>Citric Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7</td>
<td>2</td>
<td>D-Gluconic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E10</td>
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<td>D-Saccharic Acid</td>
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<td></td>
</tr>
<tr>
<td>E2</td>
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<td>Itaconic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E6</td>
<td>2</td>
<td>D,L-Lactic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>2</td>
<td>Succinamic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td>2</td>
<td>D-Alanine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>2</td>
<td>L-Aspartic Acid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3.4 COD tests

2.3.4.1 Oxidation activity of ABIL and two types of aerobic activated sludge for different carbon sources

After analysing the results obtained with the Biolog tests, several CS’s were choose in order to compare degradation activities of ABIL and two types of AAS.

Seven different CS’s were placed each, in test flasks inoculated with ABIL. This set included glucose, acetate (added as calcium acetate), sucrose, maltose, peptone, nutrient broth and Tween 80. Five CS’s (glucose, sucrose, nutrient broth, peptone and L-glutamic acid) were also placed each, in different test flasks inoculated with AAS.

Oxidation activity was calculated for each type of CS, during the first hours of test. Figures 2.9 and 2.10 show the COD$_{\text{soluble}}$ concentrations for each one of the CS’s as a function of time (data for AAS type 1 is not shown).

![Figure 2.9](image)

**Figure 2.9** Evolution of the COD$_{\text{soluble}}$ content for each one of the flasks inoculated with ABIL as a function of time, following the addition of a certain amount of CS (between 150 and 400 mg COD/L).

![Figure 2.10](image)

**Figure 2.10** Evolution of the COD$_{\text{soluble}}$ content for each one of the flasks inoculated with AAS as a function of time, following the addition of a certain amount of CS (between 150 and 350 mg COD/L).
Table 2.14 presents the oxidation activities for ABIL and the two types of AAS used in the test. Only the activities concerning the same CS’s are presented. Activity was calculated considering the first 6 hours of tests for AAS and the first 2 hours of test for ABIL.

Table 2.14 Oxidation activity values for each one of the flasks inoculated with ABIL and AAS type 1 and 2. Activity was calculated considering the test period indicated in the table.

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>AAS type 1</th>
<th>AAS type 2</th>
<th>ABIL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.30</td>
<td>0.11</td>
<td>0.12</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.12</td>
<td>0.12</td>
<td>0.086</td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td>0.14</td>
<td>0.17</td>
<td>0.24</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.058</td>
<td>0.056</td>
<td>0</td>
</tr>
</tbody>
</table>

2.3.4.2 Oxidation activity of ABIL and two types of aerobic activated sludge for peptone

Given the results obtained in 3.4.1, a new test was performed in order to analyse in more detail the peptone oxidation activity of ABIL and two types of AAS. A certain amount of peptone was added to each one of the three duplicates in order to produce concentrations of about 400 mg peptone-COD/L. The COD$_{\text{soluble}}$ was followed up during 6 hours but the activity was calculated considering the first 2 hours of the test. The COD$_{\text{soluble}}$ content of the control flasks (without peptone addition) was also determined and found to be 40 mg COD/L for ABIL and 10 mg COD/L for both AAS after 6 hours of aeration.

Figures 2.11 and 2.12 show the COD$_{\text{soluble}}$ profile as a function of time (data for AAS type 2 is not shown).

![Figure 2.11](attachment:image.png)

Figure 2.11 Evolution of the COD$_{\text{soluble}}$ content for each one of the flasks inoculated with ABIL as a function of time, following the addition of a certain amount of COD as peptone (between 350 and 430 mg COD/L).
Table 2.15 presents the average peptone oxidation activities for ABIL and the two types of AAS used in the test. Activity was calculated considering the first 2 hours of test for ABIL and AAS.

**Table 2.15** Average peptone oxidation activity values for each one of the flasks inoculated with ABIL and AAS type 1 and 2. Activity was calculated considering the first two hours of test.

<table>
<thead>
<tr>
<th></th>
<th>ABIL</th>
<th>AAS type 1</th>
<th>AAS type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation rate (g COD/g VSS.hour)</td>
<td>0.086±0.006</td>
<td>0.045±0.020</td>
<td>0.035±0.024</td>
</tr>
</tbody>
</table>

2.3.4.3 Oxidation activity of ABIL and aerobic activated sludge (type 2) for glucose

The glucose oxidation activity of ABIL and AAS type 2 was also studied in more detail. A certain amount of glucose was added to each one of the test flasks in order to produce concentrations of about 1000 mg glucose-COD/L. Oxidation activity was calculated concerning the first 5 hours. Given a higher substrate concentration, it was assumed that the latter would not be limiting during the first 5 h of test. The adsorption capacity of both ABIL and AAS type 2 was studied by placing autoclaved inoculum in control flasks. In this way it was possible to avoid COD_{soluble} decrease by biological oxidation. As other type of oxidation was not likely to occur, this methodology allowed the study of a hypothetical adsorption that would be seen by any COD_{soluble} decrease in the flasks containing autoclaved inocula.

Figures 2.13 and 2.14 present the COD_{soluble} profile as a function of time, for the test flasks as well as for the autoclaved controls.
Figure 2.13 Evolution of the COD<sub>solute</sub> content for each one of the flasks inoculated with ABIL as a function of time, following the addition of a certain amount of COD as glucose (between 900 and 1200 mg COD/L). Plots for the autoclaved controls are also shown.

Figure 2.14 Evolution of the COD<sub>solute</sub> content for each one of the flasks inoculated with AAS as a function of time, following the addition of a certain amount of COD as glucose (between 1000 and 1200 mg COD/L). Plots for the autoclaved controls are also shown.

Table 2.16 presents the average glucose oxidation activity for the test flasks. Even though the standard deviation value was high it was possible to find a clear difference in terms of the determined activity values for ABIL and AAS.

Table 2.16 Average glucose oxidation activity values for each one of the flasks inoculated with ABIL and AAS type 2. Activity was calculated considering the first five hours of test.

<table>
<thead>
<tr>
<th></th>
<th>ABIL</th>
<th>AAS type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation activity (g COD/g VSS.h)</td>
<td>0.050±0.028</td>
<td>0.021±0.020</td>
</tr>
</tbody>
</table>

2.3.5 OUR tests

These tests were performed in order to compare the oxygen uptake rate of ABIL and two types of activated sludge (type 2 and type 3) after addition of two different substrates. In figure 2.15, typical DO profiles for AAS type 3 are shown. For each profile, the test started in oxygen saturated conditions (DO in a range between 8 and 9 mg O<sub>2</sub>/L). During the first 14 minutes the
test was carried out without addition of substrate. After achieving again oxygen saturation the aeration was stopped and a specific amount of substrate was added and the DO was measured every minute for about 15 minutes. OUR$_{endo}$ was calculated concerning the first part of the test (without substrate addition). OUR$_{TAN}$ and OUR$_{glucose}$ were determined in the second part of the test after substrate addition. Given the high OUR values obtained for AAS type 3, this sample was diluted two times was performed in order to increase the resolution of the test.

Table 2.17 shows a summary on the determined OUR values for each one of the inocula. The DO profiles obtained for both AAS samples were similar. In comparison with AAS samples, ABIL showed a low OUR$_{endo}$ (between 4 and 5 times lower than AAS) and a high OUR$_{TAN}$. The latter was not detectable for AAS. OUR$_{glucose}$ for ABIL was below the detection limit of this procedure.

Table 2.17 OUR$_{endo}$, OUR$_{TAN}$, and OUR$_{glucose}$ for each one of the inocula.

<table>
<thead>
<tr>
<th></th>
<th>Average OUR$_{endo}$ (mg O$_2$/g VSS.h)</th>
<th>OUR$_{glucose}$ (mg O$_2$/g VSS.h)</th>
<th>OUR$_{TAN}$ (mg O$_2$/g VSS.h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABIL</td>
<td>1.3</td>
<td>0</td>
<td>12.1</td>
</tr>
<tr>
<td>AAS_Comimbel</td>
<td>4.8</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>AAS_Stora Enzo</td>
<td>7.4</td>
<td>2.2</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2.15 Typical evolution of the DO (mg O$_2$/L) as a function of time, in a flask containing AAS type 3. For each one of the three profiles, OUR$_{endo}$ was calculated concerning the first 14 minutes of test. OUR$_{TAN}$ and OUR$_{glucose}$ were calculated concerning the last 15 minutes of test (from t=19 to 34 min). Green and pink lines represent DO profiles for the diluted AAS type 3.
2.4 Discussion

2.4.1 Patents Survey

The patents survey present in this report was performed with two main objectives. The first one was the evaluation of which type of protection the currently commercialized NI have. Therefore, the analysis of the presently granted patents scenario allowed the evaluation of which possibilities a new product has in terms of its possible future introduction in the market.

The second aim was to understand which possibilities of being granted, a new patent on a NI could have. In parallel with any other type of publication, a patent also contributes for the state of the art. If the group of claims contained in a patent is the only part of it having legalistic value, the entire document adds data to the current body of knowledge. In strict terms, the legalistic part of a patent allows us to understand which type of products can or can not be commercially made, used, distributed or sold in a specific market. Not only the entire patent specifications but also all the other related publications should be taken into account concerning development of new products if there is any kind of interest in granting a patent on them.

In general, because of the existing differences in terms of biotechnology patents legislation, the broader claims can be found in the USA (Stix, 2006). The analysis that is presented in this text agrees with this statement. Patents that were filed by the same company (Aquaria, INC) and have a similar PCT application claiming for organisms having at least 96.1% similarity to a given 16S rRNA sequence were granted in Germany, USA and Australia. However, after the analysis of the ISA, the patent that was granted in Germany contains claims for organisms having at least 99% similarity to a given 16S rRNA sequence. As a consequence of the legislation of each country, broader claims that can be found in the patent application get narrow afterwards, in order to allow granting. Even though any type of summary concerning this issue can easily have some lack of rigour, would be true to state that:

1. In the USA and Australia, any type of NI for application in aquaria or aquaculture, containing a *Nitrospira*-related organism having 96.1% or more similarity to a given set of 16S rRNA sequences is already patent protected. All the types of NI are included (comprising not only the claimed NOB but also any other types of heterotrophic or AOB).

2. In Germany, Austria and Mexico, any type of NI for application in aquaria or aquaculture, containing a *Nitrosomonas-* or *Nitrosospira*-related organism having 99% or more similarity to a given set of 16S rRNA sequence is already patent protected. All the types of NI are included (comprising not only the claimed AOB but also any other types of heterotrophs or NOB). In Germany, also NI containing heterotrophic nitrifiers and denitrifiers are also protected if formulated in a molded article by injection.
3. In Belgium, any liquid NI formulated with CaCO₃ as a carrier and growth matrix, for use in aquatic environments is patent protected.

All of the three previous points protect different products that have been commercialized for different periods of time. In particular, point 1 refers to Biospira in terms of its NOB. Point 2 refers to Biospira in terms of its AOB and also to Baktinetten in terms of its formulation and heterotrophic denitrifiers. Point three refers to ABIL, mainly in terms of its formulation and application.

In order to be commercialized, a hypothetical new NI should not be overlapped by the already existing claims. In order to grant a patent on a new NI, the previous condition is still valid plus the need of incorporate an inventive step. The quality of the inventive step will be then measured as a function of the existing patents, other publications and other products in the market (even without patent protection).

2.4.2 Specific nitrifying activity of the NI

The ammonium oxidation activity of ABIL is probably dependent on several factors such as temperature, pH, phosphorous concentration, substrate concentration and others. After the tests that were performed it was possible to obtain indications about some variables that can have an influence in activity. The minimum and maximum observed activities were respectively 0.086 ± 0.052 (2.3.2.1) and 0.644 ± 0.018 g N-TAN/g VSS.d (2.3.2.6). The minimum value was obtained without phosphorous addition, at a moderate temperature (between 18 and 22°C) and immediately after the addition of one pulse of TAN. At the same time no previous re-activation of the stored inoculum was performed.

After adding phosphorous at a concentration of 1 mg P/L (as KH₂PO₄), it was possible to achieve the highest activity value, under the same operation conditions. A higher activity value of about 0.249 g N-TAN/g VSS.d (2.3.2.1) was also obtained after the first pulse of ammonia in a buffered solution with a concentration of about 0.62 mg P/L.

The most common application of this inoculum (domestic aquaria or aquaculture systems) are subjected to a constant load of TAN, generated by feed decomposition and excreted by fish. In order to simulate these conditions, a test was performed by adding several TAN pulses. The average ammonium oxidation rate increased from 0.256±0.016 g N-TAN/g VSS.d during the first pulse to 0.582±0.093 g N-TAN/g VSS.d during the third pulse.

The observed nitrite oxidation activities were obtained in the same test and found to be between 0.103 ± 0.052 and 0.313 ± 0.052 g N-NO₂⁻/g VSS.d (2.3.2.2). The minimum and maximum activity values were obtained respectively during the first and second nitrite pulses. Both TAN and NO₂⁻ activity values of ABIL as well as the general increasing activity trend observed after three pulses of TAN agree with previous publications (Grommen et al, 2002; 2005).
With respect to the other tested NI, all of them presented always significantly lower activities or even no activity at all. In particular, it was not possible to find any activity in the JBL product; FilterStart. Even though this product is advertised as containing living bacteria (non-specified) no special type of cold storage is recommended.

In the same way, for Biospira, only the test flask with the highest overdosage presented a noticeable activity of about 0.002 g N-TAN/dosage.d that could however also be the consequence of ammonia stripping (higher stripping than the control) since no nitrite oxidation activity was found in any of the test flasks.

BioSpira was delivered by regular post (in an isolated box with cooling gel). Therefore low activity values can probably be explained by unfavourable transport conditions.

The only observed activity for Baktinetten was found after the second pulses of either TAN or NO$_2^-$. Observed oxidation activities for TAN and NO$_2^-$ were respectively $0.011 \pm 0.016$ g N-TAN/g VSS.d and $0.025 \pm 0.004$ g N-NO$_2^-$/g VSS.d.

Product A presented higher activities than Baktinetten but about ten times lower than ABIL. TAN oxidation activity was found to be in the range $0.020 \pm 0.012$ g N-TAN/g VSS.d and NO$_2^-$ oxidation activity in the range $0.025 \pm 0.004$ g N-NO$_2^-$/g VSS.d.

Considering the set of tested products, Baktinetten and Product A were the only immobilized in a calcium alginate matrix. However, with respect to most of the applications of these inocula there is no need to recover the same. By delivering the nitrifying suspension without immobilization it would be possible to increase the activity only.

### 2.4.3 Biolog and COD tests

Biolog approach was used as a potential simple way (non-molecular) that could be used to distinguish the enriched culture of nitrifiers ABIL from a regular aerobic activated sludge. According to previous molecular studies performed with ABIL, its limited diversity in terms of nitrifiers is accompanied by a diverse heterotrophic community (Vanparys, 2006). Other studies revealed a high level of heterotrophs in enriched nitrifying communities (Burrel et al, 2001). Indeed, some heterotrophs are able to mineralize different organic compounds released by nitrifiers (Kindaichi et al, 2004).

The Biolog tests showed that the average absorbance signal obtained with the GN2 plates can be slightly higher for AAS. After 72 h of incubation, the average signal was about 20 % higher than the signal obtained after inoculation with ABIL.

After analysis of the most commonly consumed CS’s it was possible to conclude that about 50 % of the CS’s with high frequency of oxidation (as defined in 2.2.4) were the same for ABIL and for the considered sample of AAS.
In the same way as observed with the Biolog approach, COD tests showed the ability of ABIL to oxidize a diverse set of CS with an oxidation activity as high as or even higher than the activity found for AAS. Peptone oxidation activity of ABIL was equal to 2.1 ± 0.144 g COD/g VSS.d, approximately two times more than the activity determined for AAS. In the same way ABIL’s glucose oxidation activity was more than two times higher than the one determined for AAS.

The adsorption test carried out with ABIL and AAS showed that the COD content of the autoclaved samples was constant along the time. In face of this, it was possible to conclude that the determined activities corresponded to a biological oxidation.

2.4.4 OUR test

In general it was possible to conclude that OUR$_{endo}$ and OUR$_{glucose}$ were always higher for AAS. Both samples of the sludge revealed a similar behaviour giving the obtained OUR values in the same range.

After adding glucose to a sample of ABIL it was not possible to observe a higher decrease of the DO that was kept close to the oxygen saturation value. In the same way, after adding TAN as NH$_4$Cl to the AAS, the noticed DO decrease was close to the one related with the OUR$_{endo}$.

Nitrifying activity tests with ABIL showed a maximum nitrifying capacity between 0.100 and 0.650 g N-TAN/g VSS.d. This value is about 10 times higher than the reference values for the nitrifying activity obtained in WWTP.

ABIL was obtained by gradual enrichment starting from activated sludge. Indeed several studies confirmed the diverse heterotrophic community of this inoculum. The COD tests presented in this text showed high oxidation activities for ABIL, given different carbon sources. OUR tests appeared as a simple way to distinguish regular AAS from ABIL mainly supported by the existing difference between the ratios OUR$_{TAN}$/OUR$_{glucose}$.
3 Start up of an Oxygen-Limited Nitrification Denitrification (OLAND) lab scale rotating biological contactor (RBC)

Abstract

In this study, the start-up of a lab-scale OLAND RBC was examined. After 221 days of operation, a maximum removal rate of 2092 mg N/L.d or 6261 mg N/m².d was reached. A maximum removal efficiency of about 91% was observed.

During six operational periods, different supplements were added to the influent and several operational variables were manipulated. The influence of adding hydroxylamine as well as compost extracts and humic acids were investigated.

The reactor achieved the highest removal rates while operating at 0.26±0.10 mg O₂/L and pH 8.0±0.1 while adding humic acids at 25 mg/L. High nitrite concentrations up to 200 mg N-NO₂⁻/L did not cause irreversible inhibition.

Successful inhibition of NOB was achieved by hydroxylamine addition at an influent concentration of 50 – 65 mg N-NH₂OH/L.

Resumo

Neste estudo, o início da operação de um RBC (rotating biological contactor) a operar à escala laboratorial em condições OLAND (oxygen limited autotrophic nitrification denitrification) foi analisado. Depois de 221 dias de operação foi possível atingir uma taxa máxima de remoção de azoto de 2092 mg N/L.dia ou 6261 mg N/m².dia. Uma eficiência máxima de remoção de cerca de 91 % foi registada após 213 dias de operação.

Seis diferentes momentos operacionais foram identificados, durante os quais se adicionaram diferentes suplementos ao influente e se manipularam algumas variáveis operacionais. A influência da adição de hidroxilamina, bem como da adição de infusão de Dranco e de ácidos
húmicos (AH) foi também estudada. As mais altas taxas de remoção foram atingidas pelo reactor enquanto o mesmo operava a uma concentração de oxigênio dissolvido e pH de respectivamente 0.26±0.10 mg O₂/L e 8.03±0.10 e com uma concentração de ácidos húmicos de 25 mg AH por litro de influente. Altas concentrações de nitrito até 200 mg N-NO₂/L não provocaram aparentemente nenhum tipo de inibição irreversível.
Foi possível inibir com sucesso as bactérias oxidantes de nitrito através da adição de hidroxiilamina ao influente, em concentrações entre 50 e 65 mg N-NH₂O por litro de influente.

*Palavras-chave*: RBC; OLAND; Anammox; biofilme; AOB; NOB; AnAOB

### 3.1 Introduction

The removal of nitrogen from wastewater has become an important part of the wastewater treatment process due to the more stringent legislation because of the nitrogen impact in the environment. Biological nitrogen removal is effective, inexpensive and it has been adopted widely.

Conventional nitrogen removal processes used in low nitrogen loaded sewage (typical ratio C:N = 7) require two biological steps: autotrophic nitrification (to convert TAN to nitrite or nitrate) and heterotrophic denitrification (to convert the oxidized nitrogen during nitrification to dinitrogen gas). As nitrification requires high levels of oxygen (> 2 mg/L) (Third, 2002) and denitrification requires the absence of oxygen, conventional nitrification-denitrification systems carry out the two steps separately. Simultaneous nitrification and denitrification processes occur in the same vessel in similar operational conditions. Biological and physical characteristics support this process, namely the oxygen diffusion limitation inside a floc that generates anoxic zones inside the same.

In general, heterotrophic denitrification can be achieved when a ratio C:N of 7 is present. For lower ratios, only incomplete denitrification will be achieved. By adding extra supplies of carbon is possible to increase the level of denitrification. However many wastewaters are used to have low ratios C:N (< 1) making the addition of exogenous COD unviable economically (Third, 2002).

Recently, several new processes for N-removal have been developed to treat waters with low C:N ratios.

The SHARON process was the first process being successfully applied in full scale for the treatment of waters with high TAN concentration. Currently, six full-scale SHARON reactors are known to operate in stable conditions. As mentioned above, the typical SHARON process has the same biological support than other conventional nitrification-denitrification processes.

The discovery of the Anammox process was a landmark for the development of new approaches. The Anammox process is the microbiological conversion of TAN and nitrite to dinitrogen gas in the absence of organic carbon.
The SHARON process allows savings of about 40% of all the exogenous COD that would be added to carry out complete denitrification. Moreover, processes such as the “half-SHARON” plus Anammox, the complete autotrophic nitrogen removal over nitrite (CANON) or the oxygen-limited autotrophic nitrification/denitrification (OLAND) allow complete denitrification without the need of exogenous COD.

All these processes make use of AnAOB to perform the desired anammox conversion. Namely CANON and OLAND are carried out in one single reactor. Hydroxylamine is known to be a toxic intermediate in nitrification by AOB. Some studies revealed that in the presence of 42 mg N-NH₂OH/L no nitrite oxidation was observed. Addition of a lower dose of about 2.5-5 mg N-NH₂OH/L was shown to promote nitrite accumulation. This inhibitory effect of hydroxylamine was also found to be irreversible. At the same time hydroxylamine appeared to accumulate at high TAN concentrations, low DO and high pH, such as the environment promoted during this experiment.

In several WWTP, stable communities of Anammox were consistently detected in high numbers (Schmid et al, 2003). In some of these environments it is also possible to find appreciable concentrations of humic acids. It was hypothesized that these compounds could act as electron shuttles between both species of AnAOB and AOB present in the biofilm. Compost was obtained from the Dranco process (Organic Waste Systems, Belgium). Through this process is possible to convert solid biodegradable organic wastes into biogas and stabilized compost (also marketed as Humotex). This compost is rich in humic acids as a result of the microbial transformation of biomolecules.

The objective of this study was to analyse the start-up period of an OLAND process, operated in a RBC. The influence of adding hydroxylamine as well as Dranco compost extract and humic acids (HA) was evaluated. Some insights concerning the hypothetical optimal operational conditions in terms of DO and pH are also presented.

### 3.2 Materials and methods

#### 3.2.1 Lab scale reactor

The RBC used in these experiments had one axis with two groups of 20 discs. The discs were separated by an interspace of 4 mm. After developing the biofilm for 221 days, the interspace between discs was reduced to 1 mm. Determination of the total area was performed considering an interspace of 2 mm and the area of each disc in between the internal and the external diameter (external diameter = 16 cm; internal diameter = 5.5 cm; thickness (considering an interspace of 2 mm) = 3 mm). The total working area was determined according to equation 19 and found to be equal to 1.42 m²:
In equation 19, \( d_i \) and \( d_e \) respectively represent the internal and external disc diameter, and \( n \) and \( l \) respectively represent the number of discs and the disc thickness.

Figure 3.1 presents the used reactor set-up.

On one side of the reactor, an effluent overflow was installed at a fixed height of 10 cm. Through a horizontal siphon system it was possible to adjust the water level in the reactor, and thus the total working volume. The total volume was equal to 3.9 L in the first four operational periods and equal to 4.25 L in the last two operational periods. As a consequence, 50% of the discs were submerged in the wastewater in the first 4 operational periods. In the last 2 operational periods, a value of about 67 % of the discs, were submerged. The rotation speed of the discs was kept constant at 3 revolutions per minute (rpm) over the course of the experiments. The influent was dosed on one side of the reactor and the flow in the reactor was perpendicular to the axis of rotation of the discs (Figure 3.2).
Figure 3.2 Rotating biological contactor immediately after inoculation. Some of the biomass is visible in the interspace between the discs.

The reactor was kept at a fixed temperature of 34 ± 1 ºC to ensure mesophilic conditions. Reactor performance was evaluated in terms of N-removal efficiency calculated as:

\[
\frac{N_{in} - N_{out}}{N_{in}} \times 100
\]

(20)

\(N_{in}\) and \(N_{out}\) (mg N/L) represent respectively, the total concentration of nitrogen in the influent and in the effluent. Loading and removal rates were also determined per unit of volume and area according to:

\[
B_v = \frac{N_{in} \cdot Q}{V}
\]

(21)

\[
R_v = \frac{(N_{in} - N_{out}) \cdot Q}{V}
\]

(22)

\(B_v\) and \(R_v\) represent respectively, the volumetric nitrogen loading rate and the volumetric nitrogen removal rate. \(V\) (L) represents the volume of the reactor and \(Q\) (L/d), the flow. In this text, both rates are given in mg N/L.d. The rates per unit of area were calculated by using similar equations and substituting the volume \(V\) by the total area of the contactor as calculated in 19.

As mentioned above, the anammox denitrification produces also a certain amount of \(\text{NO}_3^{-}\). When both aerobic and anoxic processes are coupled, approximately 0.11 mol \(\text{NO}_3^{-}\) are produced per mol of removed TAN (equation 17).

The following ratio was calculated in order to find the amount of nitrate formed per amount of TAN removed.

\[
\frac{\text{NO}_3^{-}_{out} - \text{NO}_3^{-}_{in}}{\text{NH}_4^{+}_{in} - \text{NH}_4^{+}_{out} - \text{NO}_2^{-}_{out}} \times 100
\]

(23)
In a typical OLAND process free of NOB activity, this ratio should be 11%, according to eq. 17.

### 3.2.2 Synthetic wastewater

The synthetic wastewater used in the experiments was prepared with tap water and contained variable amounts of TAN (added as (NH$_4$)$_2$SO$_4$), 6.75 g NaHCO$_3$/g N (as carbon source and buffer), 0.07 g P/L (added as KH$_2$PO$_4$) and 0.05 g Nutriflok/L (Avecom NV, Ghent, Belgium). Nutriflok consists of a commercial mixture of macro- and micronutrients (Gernaey et al., 1997). The influent was added to the reactor twice per hour in a semi-continuous way. In the first four periods the flow rate was 15.6 ± 5.4 L/d, whereas the last two periods this was 11.7 ± 0.5 L/d.

### 3.2.3 Different operational periods

According to the different operational conditions six different periods were distinguished during the total operation period of 221 days (Table 3.1).

#### 3.2.3.1 Start-up phase (First operational period)

The reactor was inoculated with an active culture from another lab-scale OLAND RBC reactor. Approximately 200 g of mature biofilm (approximately 4.42 g VSS) were collected from this reactor. As seen in 1.16.2.3, a mature biofilm from an OLAND system should be constituted by AOB and AnAOB. Indeed, molecular analyses were performed in order to confirm this hypothesis. Through fluorescent in situ hybridization (FISH) observations was possible to conclude that the biofilm where the seeding sludge was collected was already enriched in the two major groups of bacteria (Pynaert, 2003).

#### 3.2.3.2 Hydroxylamine (NH$_2$OH) (Second operational period)

According to Peng and Zhu (2006), hydroxylamine exhibits acute toxicity to NOB causing build up of nitrite in a nitrifying system. In the beginning of the second operational period, 50 mg N-NH$_2$OH/L was added to the influent. This dose was later increased to 65 mg N-NH$_2$OH/L, since no NH$_2$OH was measured in the reactor. Probably due to biological or chemical degradation it was not possible to notice NH$_2$OH. The addition was stopped on day 90.

#### 3.2.3.3 Operation at low pH (Fourth operational period)

In order to evaluate the reactor’s performance at low pH, the concentration of NaHCO$_3$ in the effluent was decreased. As a consequence of a strong decrease, pH fell down to 6.7 (lowest recorded value). As a consequence of this adjustment, the average pH in the third period was about 7.4 against a value of about 7.9-8.0 registered in the other periods. The impact in the removal rate is analysed below.
3.2.3.4 Volume increase and addition of compost extracts (Fifth operational period)

In order to decrease the DO in the reactor by submerging a larger portion of the contactor, the working volume was increased on day 158, by using a horizontal siphon system. As a consequence, the total working volume was increased to about 4.25 L in periods five and six. From the day 161 to day 203, Dranco compost extract was dosed to the reactor influent. The infusion was prepared by contacting 1.5 kg of Dranco compost with 3 L of water at 34°C for 48 h. The infusion was subsequently sieved with a 0.1 mm sieve. One litre of the preparation (8.4 ± 0.6 g COD\textsubscript{total}/L) was then diluted 55 times in the influent container generating concentrations of approximately 153 ± 11 mg COD\textsubscript{total}/L.

3.2.3.5 Humic acids (HA) addition (Sixth operational period)

From day 203 on, a specific volume of a solution of HA (Acros Organics) was dosed to the reactor in order to generate a concentration of 25 mg HA/L. A solution containing 1 g HA/L was found to have approximately 0.93 g COD\textsubscript{total}/L, so the HA addition resulted in 23 mg COD\textsubscript{total}/L.

3.2.4 Chemical Analyses

Concentrations of TAN, NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} were determined according to the methodology described in 2.2.7. pH and DO were determined according to 2.2.7.5.

The determination of the TSS and VSS content of the reactor was performed twice (on day 150 and 229) by determining the TSS/VSS content of specific 6 cm\textsuperscript{2} areas in the contactor and extrapolating for the total area of the contactor. This determination was performed according to 2.2.7.4.

The COD analyses were performed to determine the COD content of Dranco compost extract and humic acids solution according to 2.2.7.4.1.

3.3 Results

A summary on the characteristics and performance on each one of the operational periods is given in Table 3.1.

3.3.1 Start-up period

The start-up period was initially characterized by the gradual increase loading rate accompanied by nitrite accumulation.
After inoculation of the reactor it was possible to notice low and instable removals. The transference of the biofilm was not able to preserve the structure of the same. As expected, only a significant amount of AOB were preserved in the transferred biomass, as reflected by the increasing concentrations of NO$_2^-$: At this moment, only AOB were able to oxidize their substrate to nitrite. During the first 50 days of operation, AOB were able to oxidize most of the TAN introduced in the reactor. The loading rate was increased more than ten times, from about 220 mg N/L.d to a maximum value of 2437 mg N/L.d.

The low amount of NOB present in the first days of operation was confirmed by the low nitrate concentrations, always below 100 mg N-NO$_3^-$/L during the first 50 days.

Some problems with the feeding pump were noticed on day 63. Consequently, almost all of the TAN was completely oxidized to nitrate.

However, in the end of the first period, nitrate concentration started to increase confirming the development of NOB clearly noticeable after 50 days of operation.

In the first operational period, concerning DO, no data was collected. In general, the DO was kept as low as possible all over the course of the experiment in order to inhibit NOB growth given their lower affinity for oxygen than AOB. At the same time, a low DO in the reactor avoids penetration of oxygen in the inner part of the biofilm promoting suitable conditions for the growth of AnAOB. Concerning this parameter, was possible to distinguish two different moments of operation. The first moment (operational periods 2, 3 and 4) was characterized by an average DO of 0.80 ± 0.19 mg O$_2$/L. For the second moment (operational periods 5 and 6) was possible to record an average DO of 0.31 ± 0.10 mg O$_2$/L. This difference was probably a consequence of the volume increase in the reactor. The volume change was performed in the beginning of the fifth operational period. At the same time, given the volume increase, a bigger part of the discs surface became submerged.

### 3.3.2 Hydroxylamine addition and third period of operation.

In order to prevent out-competition of AnAOB by a growing population of NOB, on day 76 hydroxylamine start being dosed to the influent. It was possible to notice a suddenly decrease on the nitrate concentration and subsequently, an accumulation of nitrite. The removal rate remained instable and only slightly higher in average, when compared with the first period. Hydroxylamine addition stopped on day 90.

In the third operational period it was possible to observe a consistently low concentration of nitrate. At the same time, nitrite concentration decreased suddenly from day 117 to day 121 and the removal rate started increasing. A period of 19 days was considered to adapt a trend line, in order to express the removal rate as a function of the time between days 111 and 130. The following expression was obtained:
\[ R_v = 0.31e^{0.0626d} \quad (111 < d < 130) \] (24)

After applying the first derivative to equation 24, a growth rate equal to \(0.063\, \text{d}^{-1}\) was obtained.
<table>
<thead>
<tr>
<th>Periods</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>0-76</td>
<td>77-90</td>
<td>91-135</td>
<td>136-157</td>
<td>158-201</td>
<td>202-222</td>
</tr>
<tr>
<td>Main changes</td>
<td>Start operation</td>
<td>Hydroxylamine addition</td>
<td>Normal operation</td>
<td>Operation at low pH</td>
<td>Reactor volume increase; addition of compost extract</td>
<td>Humic acids addition</td>
</tr>
<tr>
<td>pH</td>
<td>*</td>
<td>*</td>
<td>7.9±0.1</td>
<td>7.4±0.3</td>
<td>7.9±0.1</td>
<td>8.0±0.1</td>
</tr>
<tr>
<td>DO (mg O₂/L)</td>
<td>*</td>
<td>*</td>
<td>0.9±0.1</td>
<td>0.6±0.1</td>
<td>0.3±0.1</td>
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</tr>
<tr>
<td>Rotation speed (rpm)</td>
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<td></td>
<td></td>
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<td>HRT (h)</td>
<td>5.9±1.1</td>
<td>6.0±0.2</td>
<td>6.1±0.7</td>
<td>8.0±0.8</td>
<td>8.0±0.3</td>
<td>8.1±0.3</td>
</tr>
<tr>
<td>Bv (mg N/L.d)</td>
<td>1660±465</td>
<td>2121±128</td>
<td>1694±197</td>
<td>1535±211</td>
<td>1735±182</td>
<td>2081±161</td>
</tr>
<tr>
<td>Bs (mg N/m².d)</td>
<td>4969±1391</td>
<td>5824±351</td>
<td>4651±541</td>
<td>4217±578</td>
<td>5192±544</td>
<td>6230±483</td>
</tr>
<tr>
<td>Suplement/Concentration (mg/L influent)</td>
<td>-</td>
<td>Hydroxylamine (50 – 65 mg N-NH₂OH/L)</td>
<td>-</td>
<td>-</td>
<td>Compost extract (153 ± 11 mg COD total/L)</td>
<td>Humic Acids (25 mg HA/L)</td>
</tr>
<tr>
<td>Rv (mg N/L.d)</td>
<td>186±234</td>
<td>420±158</td>
<td>534±429</td>
<td>550±73</td>
<td>1118±336</td>
<td>1828±144</td>
</tr>
<tr>
<td>Rs (mg N/m².d)</td>
<td>556±700</td>
<td>1154±433</td>
<td>1467±177</td>
<td>1511±199</td>
<td>3348±1005</td>
<td>5471±431</td>
</tr>
<tr>
<td>N removal (%)</td>
<td>4.2±5.1</td>
<td>19.6±6.6</td>
<td>32.3±25.7</td>
<td>36.1±4.6</td>
<td>63.8±14.8</td>
<td>87.9±2.3</td>
</tr>
<tr>
<td>NO₂⁻ effl concentration (mg N-NO₂⁻/L)</td>
<td>165±65</td>
<td>231±63</td>
<td>129±94</td>
<td>77±31</td>
<td>90±46</td>
<td>8±2</td>
</tr>
<tr>
<td>N-NO₂⁻ formed/N-NH₄⁺ removed (%)</td>
<td>60±50</td>
<td>49±11</td>
<td>56±41</td>
<td>53±6</td>
<td>16±10</td>
<td>8±1</td>
</tr>
</tbody>
</table>
3.3.3 Operation at low pH

In order to evaluate the reactor’s performance at a low pH, the concentration of NaHCO$_3$ was decreased. As a consequence of the lower buffer capacity, protons produced during TAN oxidation to nitrite were able to produce a stronger decrease than initially desired. pH fell down to 6.7 on day 139. A sharp and suddenly decrease in the removal rate accompanied by accumulation of nitrite were noticed. In this fourth period, the inflow was also adjusted in order to increase the HRT from about 6 to 8 hours. The loading rate was kept around the same value by increasing the TAN concentration in the influent.

3.3.4 Volume increase and Dranco compost extract addition

After stabilizing the pH value around 8.0, the working volume of the reactor was increased from 3.9 to 4.25 L, on day 158. It was possible to notice an immediate effect in the DO. The average DO value that had been close to 1.0 mg O$_2$/L decreased to an average value of 0.33 and 0.26, respectively in the fifth and in the sixth period. In the beginning of this operational period, the VSS content of the reactor was determined and found to be equal to 42 ± 2 g VSS (ratio VSS/TSS = 0.68).

Addition of Dranco compost extract started on day 161. From this moment on, till the end of the fifth period it was possible to observe a stable increase of the removal rate. At the same time, nitrate concentration was kept in low values confirming the successful NOB inhibition. No accumulation of nitrite was verified during this period.

As performed previously in 3.3.2, one period of time was considered in order to adapt one trend line. An exponential trend line was obtained for the period of time between day 147 and 221 (Figure 3.5). The following expression was obtained:

$$ R_v = 33.924 e^{0.019d} \quad (147 < d < 221) \quad (25) $$

Therefore, a growth rate of 0.019 was obtained for equation 25.

3.3.5 Humic acids addition

In the last period of operation, instead of Dranco compost extract, a specific volume of a concentrated solution of HA was added to the influent container in order to produce concentrations of about 25 mg HA/L (approximately 23.5 mg COD/L). This period was characterized by the same positive trend already verified in the fifth period. A trend line was adapted to the period of time between day 201 and 221 and a removal growth rate of 31 mg N/L.d$^2$ was recorded. A maximum removal rate value of 2092 mg N/L.d or 6261 mg N/m$^2$.d was obtained on day 221. A maximum removal efficiency of 91% was verified on day 213. In the
end of this operational period, the VSS content of the reactor, was found to be equal to 65 ± 12 g VSS (ratio VSS/TSS = 0.73).

3.3.6 Removal efficiency

The following ratio was used to calculate the removal efficiency:

$$\frac{R}{B} = \frac{N_{in} - N_{out}}{N_{in}} \times 100$$

(26)

Figure 3.4 shows the evolution of the removal efficiency against time.
The graph represented by Figure 3.4 shows clearly the trend of efficiency removal increase. However, in the last days of this test it was noticeable the attainment of a steady state around 95 % removal efficiency.

### 3.4 Discussion

During the first period of operation it is possible to distinguish two different moments. The first one (day 0 to day 35) is characterized by a low constant loading rate and a low AOB activity. From day 35 on, almost all the TAN started to be converted to nitrite, probably as a consequence of AOB growth. The feeding pump stopped for some hours on day 63 as can be confirmed by the total conversion of ammonia to nitrate. After day 63, till the end of the first period it was possible to observe a growing NOB activity. At the same time, a thin brownish biofilm started to develop. In this early stage of its development the biofilm was probably not able to create an anoxic environment suitable for AnAOB.

The addition of hydroxylamine was able to inhibit the activity of NOB. However, at least 30% of their activity was retained as it's possible to observe by the lower but existing nitrate production. In this second period of operation, a higher removal rate was also observed. However, it is unsure if AnAOB were responsible for this removal, given its low growth rate and vulnerability towards oxygen. Possibly chemical denitrification in the presence of NH₂OH contributed to the observed nitrogen removal.
As reported previously by Kuai and Verstraete (1998), *Nitrosomonas* sp. are able to produce dinitrogen gas under oxygen-limiting conditions. Therefore, the observed removal rates in this period (up to 674 mg N/L.d) could be explained by a combination of factors. Given an average pH close to 8.0 and a temperature of about 34 °C, ammonia stripping could also be responsible for a significant part of all the nitrogen removal. The nitrite concentration in the influent achieved its maximum value in this period (302 mg N-NO$_2$/L).

After stopping hydroxylamine addition it was possible to maintain NOB inhibition. As hypothesised previously, the achieved hydroxylamine inhibition was kept all over this 47 days period.

For the very first time, on the third operational period, removal rate started to increase at an approximate growth rate of 0.063 d$^{-1}$. At this point, the low nitrite accumulation and the relatively low nitrate production was probably accompanied by the start up of AnAOB activity. Visually, in the end of the third period it could be seen a thicker brownish biofilm spread all over the contactor forming a homogenous layer.

According to the constant growth observed for the removal rate, it was also possible to observe (Figure 3.5) a stable growth of the removal efficiency, approximately from day 110 to the end of the third period (day 137).

According to Jetten and Strous (1999), nitrite concentrations higher than 180 mg N-NO$_2$/L would inhibit AnAOB activity. However, it seems that nitrite concentration fell suddenly in the second half of the third period due to the rapidly increasing removal efficiency. Probably, AnAOB subjected to high concentrations of nitrite for several days were able to deal with it. Considering the stoichiometry of equation 17, approximately 11% of all the removed TAN should be converted to nitrate by Anammox. Despite a higher value in the first half of the third period, a ratio equal to 29 ± 8% was found between day 117 and 135.

Probably, after 137 days of operation, a mature OLAND biofilm with anoxic zones was formed. However, an induced pH decrease from 8.0 to 7.3 had an immediate effect on the removal rate that fell down from 906 to 300 mg N/L.d at day 136.

The fourth operational period was then characterized by an average removal rate of 550 ± 73 mg N/L.d. The removal efficiency fell to values below 40% before starting to recover from day 150 on. Although there were no irreversible inhibition reported for AOB or AnAOB for pH 6.7 (lowest value recorded), only 35 days later, in the fifth operational period it was possible to notice again the same removal efficiencies.

After increasing the working volume to 4.25 L, the bulk DO concentration decreased from 0.62 ± 0.08 mg O$_2$/L in the fourth period to 0.33 ± 0.10 mg O$_2$/L, in the fifth. Probably, given the higher volume of water and a larger part of the discs that became submerged, AOB’s oxygen uptake was enough to decrease the DO. The dosing of Dranco compost extract was performed in parallel with the volume increase. Therefore it’s possible to conclude that both effects when
taken together had a neutral to positive impact. At the same time, during the fifth period, the biofilm started to increase its thickness, developing optimal conditions for AnAOB growth.

The humic acids dosing started immediately after stopping adding Dranco compost extract to the influent, till the end of the sixth period. It was possible to notice the same growth trend of the removal rate as previously observed. This trend was kept without slowing down till the end of this last operational period. At the same time, nitrite levels fell to the lowest levels ever achieved and the average ratio nitrate formed per nitrogen removed had a value of 8 ± 1%, even lower than the initially predicted. The highest removal rate was achieved on day 221 (2092 mg N/L.d or 6261 mg N/m².d). The highest removal efficiency (91%) was also achieved during this period. In face of these results it’s possible to conclude that HA addition at a dosage of 25 mg/L_{inu} had a positive to neutral effect.
Production of an inoculum in a concentrated medium and analysis of its activity after freezing and exposure to a de-icer

Abstract

Accumulation of ice in certain vulnerable environments such as roads and airports is potentially dangerous contributing strongly for accidents in face of freezing conditions and precipitation. De-icing techniques are used in order to avoid accumulation of ice in these environments. By spraying an ice layer with a de-icer is possible to thaw it. The de-icers consist normally of concentrated aqueous solutions able to decrease the freezing point of water and thus, able to thaw the ice. Even when non-toxic solutes are used, the accumulation of de-icers in nature is not desirable, given its high COD content.

In this study, an inoculum was enriched from activated sludge. This inoculum was subsequently frozen and mixed with a commercial de-icer. After freezing and subsequently melting the enriched inoculum it was possible to retain up to 60 % of the original activity. In the same way, after 60 minutes of direct contact with the concentrated de-icer it was possible to preserve the activity of the inoculum.

In this way it was shown that is possible to mix a frozen inoculum with a commercial de-icer in order to accelerate the degradation process of the same after application.

Keywords: Activated sludge; De-icer

Resumo

A acumulação de gelo em determinados ambientes considerados vulneráveis, tais como estradas e aeroportos, é potencialmente perigosa contribuindo fortemente para a ocorrência de acidentes face a condições de baixas temperaturas e precipitação. Técnicas de descongelamento são usadas por forma a evitar a acumulação de gelo neste tipo de ambientes. Através da dispersão de um descongelante numa camada de gelo já formada é possível fundi-la. Os descongelantes consistem normalmente em soluções aquosas
4.1 Introduction

De-icing is the process by which ice is removed from a surface. Apart from mechanical methods and the application of heat, the addition of chemicals to decrease the freezing point of water can also be used. According to the law of Raoult, the addition of a solute to liquid decreases the tendency for that liquid to become a solid or a gas. For example, as a consequence of this, by adding a solute to water, the freezing point will decrease below its normal value (0 °C). The vapour pressure of a solvent in solution is equal to its mole fraction times its vapour pressure as a pure liquid. The freezing point depression is thus proportional to the molality of the solute. Freezing point depression occurs whenever a solute is added to a pure solution such as water. The solute molecules disrupt the ability of the solvent to form crystals during the freezing process (Ebbing, 1990).

Several companies produce de-icers that can be used in airports or regular roads. The de-icing process is usually done by spraying a certain surface with a de-icing fluid containing a concentrated solute (usually monopropylene glycol, ethylene glycol or acetate).

The de-icer used in this test was developed for airport applications. By accumulating ice in the surface of aircraft’s wings, the ability to fly in stable conditions can be diminished. De-icing is thus performed to avoid water freezing in the wings or in other parts in the outside of the airplane.

The application of large amounts of de-icers in airports and public roads can have negative effects in nature. Even biodegradable compounds such as acetate, when present in large amounts in aquatic systems are able to increase the COD of these systems, generating lack of oxygen for other living species.
The aim of this test was to investigate whether such an inoculum could be or not produced and stored at low temperatures for subsequent uses in real conditions. After storage, the inoculum could be directly applied to surfaces after de-icer application or mixed with the de-icer some moments before application. The extra COD load generated by the de-icer (and also by the residual COD in the solution of inoculum) could be subsequently degraded.

In this study, an inoculum was produced starting with an activated sludge culture. A certain amount of activated sludge was fed with glycerol in order to increase the biomass content. Afterwards the activity retention of the produced inoculum was analysed as a function of the residual concentration of glycerol. The inoculum was also exposed to a certain amount of a commercial de-icer and the activity was measured after exposure. Several tests were performed in order to optimize the test procedure. A complete test for two different inocula is presented in this study.

4.2 Materials and methods

4.2.1 Production of the inoculum

In order to produce this inoculum, Erlenmeyer flasks were inoculated with 1000 mL of aerobic activated sludge type 2 (3.2 g VSS/L) as described in 2.2.5.1. An appropriate volume of a glycerol solution was added in order to produce COD concentrations of about 15 g COD-glycerol/L. The medium was also supplemented with N (500 mg N/L added as NH₄Cl), P (100 mg P/L added as KH₂PO₄) and Nutriflok (200 mg/L).

The flasks containing the medium were placed in a shaker at 28ºC with appropriate aeration assured by air blowing. The DO was kept above 3 mg/L (minimum value determined by decreasing visually the air flow without stopping agitation). The pH decreased from 7.7 to 7.3 during the test. Samples were collected at t = 0, 24, 48, 72 and 96 h, centrifuged 10 min at 3000 RPM and paper filtered. The COD content of the flasks was followed and the VSS content in the end of the test was determined.

4.2.2 Activity analysis after freezing

72 (AAS_1) or 96 h (AAS_2) after the beginning of the test, the inoculum was frozen in small cubes at -20ºC and kept for 7 days. After this period of time, a volume equal to one litre was unfrozen by placing the small cubes in a flask at 34ºC with gentle stirring.
In order to determine the activity of the frozen sludge the test flasks were placed in a shaker at 28°C with appropriate aeration assured by air blowing. DO was kept above 3 mg/L and pH kept above 7.2.

No COD was added to the previously frozen sludge as a certain concentration of glycerol was kept till the freezing moment. An amount of about 6 g COD-glycerol/L was added to the regular sludge. COD of both flasks was followed for 48 h (samples were collected at t=0, 3, 6, 24 and 48 h, centrifuged 10 min at 3000 RPM and paper filtered).

### 4.2.3 Activity analysis after exposure to the de-icer

The de-icer was obtained from Proviron (Belgium) and consisted in a concentrated solution of potassium acetate and an unknown concentration of corrosion inhibitors developed for application in airports.

After keeping the sludge at -20°C for 7 days two different methods were followed:

1) A volume of about 25 mL of sludge was unfrozen at 36°C by contacting it with 25 mL of de-icer at 36°C for one hour. This mixture was afterwards diluted in 950 mL of tap water and placed in a flask with agitation and air blowing.

2) A volume of about 25 mL of sludge was unfrozen at 36°C by diluting it in 1000 mL of a solution containing 25 mL of de-icer and tap water. This solution was placed in a flask with agitation and air blowing.

The COD content of the flasks was followed for 48 h (samples were collected at t=0, 24 and 48 h and filtered with 0.45 µm syringe filters). The flasks were placed in a shaker at 28°C with appropriate aeration assured by air blowing. DO was kept above 3 mg/L and pH kept above 7.2.

### 4.3 Results

#### 4.3.1 Production of the inoculum

After a 72 or 96 h incubation at 28°C, the VSS was found to be respectively 5.25 or 5.54 g VSS/L.

Therefore, the average yield was found to be equal to 0.40 ± 0.28 g VSS/g COD<sub>consumed</sub>. Figure 4.1 shows the typical evolution of the COD against time. An average activity of 0.664±0.1 g COD/g VSS.day was observed. Immediately after the incubation, the inocula were frozen with
different residual glycerol concentrations of about 2 and 6 g/L. In one extra day of incubation it was possible to decrease in 4 g/L the residual concentration of glycerol in the inoculum. Previous tests showed similar yields in the range of 0.3-0.4 g VSS/g COD consumed and activities up to 0.55 g COD/g VSS.day, slightly lower than the ones obtained in this test. Figure 4.1 presents the COD content evolution as a function of time, for both inocula.

Figure 4.1: Evolution of COD against time for each one of the flasks with incubation times of 72 h (AAS_1) and 96 h (AAS_2).

### 4.3.2 Activity analysis after freezing

The COD content was followed after unfreezing the inocula. The oxidation rates were calculated concerning the period of time between t = 0 and 48 h.

Figure 4.2: Evolution of COD against time for AAS_1 and 2 AAS_2.
None activity at all was observed for the AAS_2. For the inoculum with the highest residual glycerol concentration an activity equal to 0.312 was found. In previous tests it was possible to obtain activities up to 0.384 g COD/g VSS.day with a residual glycerol concentration of about 6 g/L.

4.3.3 Activity analysis after exposure to the de-icer

4.3.3.1 Activity analysis after exposure to the deicer (according to method 1)

A value of about 0.37 g VSS/L (AAS_1) and 0.39 g VSS/L (AAS_2) was generated as a consequence of the used dilution to perform this test. The COD content of the flasks inoculated with unfrozen activated sludge according to method 1) (for 1 and 24 h of direct contact) was followed. No COD was added in the beginning of the test. Concerning the assays realized after direct contact with the deicer for 24 hours, no activity was noticed for both inocula. Figure 3 shows evolution of COD against time for unfrozen AAS.

![Figure 4.3](E36_exposure_method_1) Evolution of COD against time for unfrozen AAS, according to method involving direct exposure to undiluted deicer.

Once again, none activity at all was observed for the AAS_2. For AAS_1, an activity equal to 0.288 was found.

4.3.3.2 Activity analysis after exposure to the de icer (according to method 2)
A value of about 0.26 g VSS/L (AAS_1) and 0.28 g VSS/L (AAS_2) was generated as a consequence of the used dilution to perform this test according to method 2 previously described. The COD of the flasks inoculated with unfrozen activated sludge according to method 2 was followed. No COD was added in the beginning of the test. Figure 4 shows evolution of COD against time for unfrozen AAS.

Figure 4: Evolution of COD against time for unfrozen AAS according to method 2 involving exposure to diluted E36.

![Figure 4](image)

None activity at all was observed for the AAS_2. For AAS_1, an activity equal to 0.190 was found.

### 4.4 Discussion

With respect to the production of the inoculum from activated sludge, an initial COD concentration of 15 g/L (added as glycerol) and a retention time of three days could be useful in order to maximize COD conversion in biomass while keeping a residual value of about 6 g COD-glycerol/L. Therefore, glycerol could act as a cryoprotectant preserving activity while freezing the inoculum.

For a residual concentration of glycerol equal to 6 g COD-glycerol/L it was possible to retain 60% of the activity. Some tests showed that a concentration as low as 2 g COD-glycerol/L could not be able to preserve any activity.

Concerning the exposure to either diluted or non diluted de-icer it was possible to notice activity retention in the presence of higher residual glycerol concentrations. Given the high COD load used in this test (higher than 45 g COD/g VSS) and the typical uncertainty
associated to COD measurements it was not possible to determine with accuracy an activity retention value. Therefore this test should be considered as a qualitative evaluation. In face of this evaluation appears to be possible to directly mix the diluted or non diluted de-icer with the frozen inoculum. A period of time up to 60 minutes could be used to contact directly the frozen inoculum with the non diluted de-icer.

Therefore, the mixture de-icer plus inoculum could be directly applied to surfaces. All the activity tests were performed at temperatures higher than 28ºC. However, it is possible to expect real working conditions below 5ºC. In real working conditions a containment system should be used to capture all the used liquid. An aerobic activated sludge system could be used subsequently to degrade the COD of the mixture, given the provided concentrated inoculum.
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