CORRELATION OF ACTIVITY MEASUREMENTS 
AND MOST PROBABLE NUMBER COUNTS 
DURING BIOAUGMENTATION OF ACTIVATED SLUDGE

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<th>Description</th>
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<td>MPN</td>
<td>Most Probable Number</td>
</tr>
<tr>
<td>STNA</td>
<td>Short Term Nitrification Assay</td>
</tr>
<tr>
<td>NBC</td>
<td>Nitrifying Bacteria Culture</td>
</tr>
<tr>
<td>RAM</td>
<td>Respiration Activity Measurement</td>
</tr>
<tr>
<td>BOD</td>
<td>Biochemical Oxygen Demand</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>sCOD</td>
<td>Soluble Chemical Oxygen Demand</td>
</tr>
<tr>
<td>TSS</td>
<td>Total Suspended Solids</td>
</tr>
<tr>
<td>VSS</td>
<td>Volatile Suspended Solids</td>
</tr>
<tr>
<td>HRT</td>
<td>Hydraulic Retention Time</td>
</tr>
<tr>
<td>CFSTR</td>
<td>Continuos-Flow Stirred-Tank Reactor</td>
</tr>
<tr>
<td>MCRT</td>
<td>Mean Cell Retention Time</td>
</tr>
<tr>
<td>MLSS</td>
<td>Mixed Liquor Suspended Solids</td>
</tr>
<tr>
<td>MLVSS</td>
<td>Mixed Liquor Volatile Suspended Solids</td>
</tr>
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</table>
1. Objectives

The goal of this diploma thesis was to study nitrification in wastewater by nitrifying bacteria with different methods. The methods used included the Most Probable Number (MPN) counting technique, the Short Term Nitrification Assay (STNA) and the Respiration Activity Measurement (RAM).

The MPN counting technique for nitrifying bacteria is one of the most commonly used methods in ecological studies of nitrification. It is a technique to estimate the number of cells involved in a particular, oxidative step indirect and statistically. The STNA and RAM are methods to measure the activity of the nitrifying bacteria in-situ directly (STNA) or indirectly (RAM).

The basic idea of bioaugmentation is that regular inoculations of biomass from a cultivated Nitrifying Bacteria Culture (NBC) with a certain activity and number of nitrifiers the reduction of ammonia in a wastewater treatment plant increases. These bacteria are added to a continuous flow activated sludge process. The primary goal of this study was to investigate different methods to quantify the nitrifying bacteria and their activity in the activated sludge reactors. Therefore different amounts of the same nitrifying bacteria culture were added to low, medium and high dose reactors. A control reactor without additions was also operated. The reactors were operated under the same conditions, except for biomass inoculum size. It was attempted to count and measure the added biomass using the above named methods.

The reactor operating conditions were established to be similar to typical full-scale wastewater treatment plants in terms of COD, TSS, VSS and ammonium concentrations in the influent and the effluent.

The first part of the study focused on cultivating the NBC, setting up all of the reactors and achieving steady-state operation.
The second part of the study focused on improving and adjusting the methods described for MPN, STNA, and RAM.

The third part of the study focused on comparing the methods and developing relationships that will be useful for mathematical simulations of the bioaugmentation process.
2. Introduction

The nitrification of ammonia proceeds normally in two steps. The first step is the oxidation of ammonia to nitrite, the second step is the oxidation of nitrite to nitrate. The first step is as follows:

$$\text{NH}_4^+ + 1.5 \text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + 2\text{H}^+$$  \hspace{1cm} \text{(Equation 1: Ammonium Oxidation, 1st step)}

It is observed that oxygen is needed and that the pH would decrease without pH control. The second step is as follows:

$$\text{NO}_2^- + 0.5 \text{O}_2 \rightarrow \text{NO}_3^-$$  \hspace{1cm} \text{(Equation 2: Ammonium Oxidation, 2nd step)}

For these two steps, different chemolithoautotrophic bacteria are responsible. The first step, the oxidation of ammonia to nitrite is the slower step (rate controlling) and therefore of greater interest. After ammonia is oxidized to nitrite, the further oxidation to nitrate is rapidly and immediately. Therefore all methods utilized are concentrated on the first step. With the Most Probable Number (MPN) technique it is possible to estimate the number of nitrifying bacteria indirectly and statistically. In order to determine MPN, samples of the culture have to be diluted several times and the dilutions have to be incubated in a prepared media for several weeks or months.

There are two requirements to obtain high counting efficiency. First, media and growth conditions must be used, which allow any and all of the nitrifying bacteria cells present to grow to a population large enough to be detected. Second, the cells present must be sufficiently separated from any particulate matter so that each cell will be individually suspended and can be transferred efficiently during the dilution process (Belser, L.W., 1979).

The determination of positive MPN tubes is based on the presence of nitrate and nitrite, the metabolism end-product of the nitrifying bacteria. Therefore the initial samples have to be free of both. To achieve this condition the samples were washed with phosphate buffer as described later.
MPN tubes are positive when the color of the medium changes from blue to yellow. The color change occurs when NH₄-N is oxidized, releasing H⁺ and decreasing the medium pH. The indicator utilized (bromothymol blue) appears blue in the neutral and basic range and turns yellow in the acid range.

To prove that the color change results from the presence of nitrate or nitrite, the positive tubes must be tested chemically for nitrate and/or nitrite. Because the color change does not always occur in the positive tubes, all tubes from the first two higher dilution with negatives tubes as well as all the negative tubes in a series with positive tubes, must also be tested for nitrite and nitrate.

The incubation time of the MPN tubes was at least six weeks. First color changes appeared approximately after one or two weeks. The MPN count was completed when in a period of two weeks the number of positive tubes did not change anymore.

The long incubation times of the MPN tests are a distinct drawback and make it impossible to get a quick idea about the present MPN. Ideally the incubation should last just long enough to account for all the inoculum cells capable of growth.

It is possible that under some conditions the MPN method underestimates an indigenous nitrifier population. One of the reasons why the MPN technique may underestimate populations is because of the requirement that all genera and strains of ammonium oxidizers have to be able to grow in one medium which is not necessarily true. Another reasons can be the pH of the medium, the NH₄⁺ concentration and the phosphate buffer used for the preparation of the dilutions. For these reasons, it is necessary to have an idea of the relative counting efficiency when MPN counts are used in a study (Belser, L.W. and E.L. Mays, 1981). It has been proposed that estimates of counting efficient could be made by making nitrifier activity measurements in addition to MPN counts (Belser, L.W., 1979). This requires that a theoretical estimate be made of the population to produce the observed activity.

The Short Term Nitrification Assay (STNA) has the advantage that results can be obtained almost immediately. To the test culture, diluted in phosphate buffer, ammonium
is added. The culture is then shaken on a orbital shaker, and samples are taken at appropriate intervals and analyzed for nitrite.

Since nitrite is produced as ammonium is oxidized, the rate at which nitrite accumulates (when the further reaction to nitrate is inhibited), is equal to the rate at which the ammonium is oxidized. The further oxidation of nitrite to nitrate can be almost completely inhibited by the addition of chlorate. Therefore only nitrite has to be measured to determine the nitrification activity. The nitrite concentration is determined by colormetric methods (Bremner, J.M. 1965).

To carry out the STNA under controlled conditions, the initial sample has to be free of ammonium, nitrite and nitrate. Therefore the mixed liquor samples are washed with phosphate buffer and centrifuged as described later.

The STNA gives a measure of the activity in-situ, before the organisms have an opportunity to increase in number.

Initial problems during the STNA included determining the necessarily conditions to get maximum activity. The STNA conditions described in the literature (Belser, L.W. and E.L. Schmidt, 1994) were found not to be optimal for the test culture. The problem was the high concentration of nitrifying organisms in the initial samples.

The mixed liquor samples had to be diluted several times and the concentration of ammonium and chlorate had to be varied as described later.

There are undefined limits to the increase of the chlorate concentration, because too high of a chlorate concentration will inhibit the ammonium oxidation reaction (Hyness, Rusell K. and Roger Knowles 1983). Under this condition, the STNA will underestimate the nitrifyer activity. However too low of a chlorate concentration will not completely stop the nitrite oxidation to nitrate and consequently activity will be underestimated.

Activity Measurements represent nitrification potential, which will be achieved in the natural habit only when the conditions are optimal for nitrification. Also these tests only measure the activity of active cells at the time of the STNA.

To compare activity measurements with cell counts it has to be noted that probably not all cells are active.
The activity of the different pure strains of nitrifiers is variable as reported by others (Belser, L.W. and E.L. Schmidt, 1994) in the following table (Table 1):

<table>
<thead>
<tr>
<th>Culture: Ammonium Oxidizers</th>
<th>Activities per cell in picomol per cell per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrosomonas europea</td>
<td>0.011</td>
</tr>
<tr>
<td>Nitrosomonas sp.</td>
<td>0.023</td>
</tr>
<tr>
<td>Nitrospira briensis</td>
<td>0.004</td>
</tr>
<tr>
<td>Nitrosolobus multiformis</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Table 1: Maximum activities per cell determined during exponential growth of nitrifiers in pure culture.

In order to use those values, one has to know which genus is mainly present. In waste water, like that examined in the present study, the predominate ammonium oxidizer is believed to be Nitrosomonas sp. with an activity of 0.023 pmol/cell*h.

Previous studies, to compare the MPN with the STNA, showed that the activity measurements were much higher than expected from the MPN count (Belser, L.W. 1979; Belser, L.W. and E.L. Mays 1981; Smorczewski W. T. and E.L. Schmidt 1991). The main reason cited for this is underestimated MPN counts. Better results can be expected from the present study, because the initial samples of all reactors are from the same cultivated Nitrifying Bacteria Culture (NBC).

The third method used in this study was the Respiration Activity Measurement (RAM). For this method, ammonium sulfate was added to mixed liquor samples in Biochemical Oxygen Demand (BOD)-flasks. To start the RAM, the oxygen concentration in the flasks was raised to more than 6 mg/l. The decrease of oxygen, under these conditions, was measured at appropriate intervals.

In a side-by-side control test, the same initial samples received ammonium sulfate (same concentration) and nitrapyrin. The same treatment and measurements follow.

Nitrapyrin is an inhibitor for ammonium oxidizers. Through the inhibition of the ammonium oxidizers the nitrite oxidizers are also inhibited indirectly, because no nitrite was produced. Therefore nitrification is completely inhibited.

The difference between the oxygen decrease in the active and control flasks is seen as the oxygen used during nitrification.

The RAM tests are carried out with and without addition of chlorate in order to find out whether the inhibition of nitrite oxidizers was complete and successful.
3. Material and Methods

3.1 Most Probable Number

3.1.1 Materials

3.1.1.1 Ammonium Oxidizer Medium

<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>Concentration of stock solution (g/100 ml)</th>
<th>Stock solution required per liter of NH₄⁺ oxidizer media</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>5.0</td>
<td>10 ml</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1.34</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>4.0</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td>0.04</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.72</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Cheated iron</td>
<td></td>
<td>1.0 ml</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.246</td>
<td></td>
</tr>
<tr>
<td>EDTA disodium</td>
<td>0.331</td>
<td></td>
</tr>
<tr>
<td>Trace elements</td>
<td></td>
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<td>Na₂MoO₄·2H₂O</td>
<td>0.01</td>
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<tr>
<td>MnCl₂</td>
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</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.0002</td>
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</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Ammonium Oxidizer Medium

1 Calcium chloride and magnesium sulfate are combined, autoclaved separately, and added aseptically to sterile solution of remaining ingredients.

- Make all of the solutions in distilled or deionized water.

- Adjust pH to 7.0 to 7.2 with 2% Na₂CO₃.

- Add 4 ml of this medium to each culture tube, cap the tubes, and sterilize for 15 min. at 121 °C.
3.1.1.2 Phosphate buffer

- Concentrations of stock solutions: 3.48 g $\text{K}_2\text{HPO}_4$/100 ml
  2.72 g $\text{KH}_2\text{PO}_4$/100 ml

- Add 4 ml of potassium monohydrogenphosphate ($\text{K}_2\text{HPO}_4$) and 1 ml of potassium dihydrogen phosphate ($\text{KH}_2\text{PO}_4$) stock solution per liter of distilled or deionized water.

- Sterilize for 15 min at 121 °C

- Transfer sterile 90 ml in each screw-capped bottle

3.1.1.3 Modified Griess-Ilosvay reagents:

Diazoting reagent: Dissolve 0.5 g of sulfanilamide in 100 ml of 2.4N HCl.

Store the solution in a refrigerator.

Coupling reagent: Dissolve 0.3 g of N-(1-naphthyl)-ethylenediamine hydrochloride in 100 ml of 0.12N HCl. Store the solution in an amber bottle in a refrigerator.
3.1.1.4 Nitrate spot test reagent

Dissolve 50 mg of diphenylamine in 25 ml of conc. Sulfuric acid (H₂SO₄). Store in glass-stoppered dropping bottles protected from light and prepare fresh solutions after 14 d.

3.1.1.5 Sodium carbonate (Na₂CO₃), 2% for initial adjustment of medium to pH 7.0 to 7.2.

3.1.2 Preparation of samples (washing)

- Centrifuge 50 ml of the mixed liquor for 20 minutes and remove the supernatant.
- Add phosphate buffer to a volume of 50 ml, centrifuge again for 20 minutes.
- Remove the supernatant, replace by phosphate buffer.
- Shake well before starting the dilutions.

3.1.3 Procedure

- Transfer 10 ml of the washed mixed liquor sample to a screw-capped bottle containing 90 ml of sterile buffer and shake vigorously (10⁻¹ dilution).
- Transfer 10 ml of this 10⁻¹ dilution to an screw-capped bottle using a sterile pipette (10⁻² dilution).
- Continue dilution to at least 10⁻⁷. If a high population is expected, make dilutions to 10⁻¹² or 10⁻¹³.
3.1.4 Most Probable Number (MPN) Table

To calculate the number of nitrifying bacteria the positive MPN tubes have to be recorded and the number of bacteria has to be calculated with the following formula:

\[
\text{MPN value} \times \frac{10}{\text{largest volume tested in dilution series used for MPN determination}} = \text{MPN} / 100 \text{ ml}
\]

Equation 3: MPN value

The MPN value can be selected from the following table (Table 3):

<table>
<thead>
<tr>
<th>Combination of Positives</th>
<th>MPN Index/100ml</th>
<th>95% Confidence Limits</th>
<th>Combination of Positives</th>
<th>MPN Index/100ml</th>
<th>95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>0-0-0</td>
<td>&lt; 2</td>
<td>-</td>
<td>-</td>
<td>4-2-0</td>
<td>22</td>
</tr>
<tr>
<td>0-0-1</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>4-2-1</td>
<td>26</td>
</tr>
<tr>
<td>0-1-0</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>4-3-0</td>
<td>27</td>
</tr>
<tr>
<td>0-2-0</td>
<td>4</td>
<td>1</td>
<td>13</td>
<td>4-3-1</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4-4-0</td>
<td>34</td>
</tr>
<tr>
<td>1-0-0</td>
<td>2</td>
<td>1</td>
<td>11</td>
<td>5-0-0</td>
<td>23</td>
</tr>
<tr>
<td>1-0-1</td>
<td>4</td>
<td>1</td>
<td>15</td>
<td>5-0-1</td>
<td>30</td>
</tr>
<tr>
<td>1-1-0</td>
<td>4</td>
<td>1</td>
<td>15</td>
<td>5-0-2</td>
<td>40</td>
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<td>1-1-1</td>
<td>6</td>
<td>2</td>
<td>18</td>
<td>5-1-0</td>
<td>30</td>
</tr>
<tr>
<td>1-2-0</td>
<td>6</td>
<td>2</td>
<td>18</td>
<td>5-1-1</td>
<td>50</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>5-1-2</td>
<td>60</td>
</tr>
<tr>
<td>2-0-0</td>
<td>4</td>
<td>1</td>
<td>17</td>
<td>5-2-0</td>
<td>50</td>
</tr>
<tr>
<td>2-0-1</td>
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<td>2</td>
<td>20</td>
<td>5-2-1</td>
<td>70</td>
</tr>
<tr>
<td>2-1-0</td>
<td>7</td>
<td>2</td>
<td>21</td>
<td>5-2-2</td>
<td>90</td>
</tr>
<tr>
<td>2-1-1</td>
<td>9</td>
<td>3</td>
<td>24</td>
<td>5-3-0</td>
<td>80</td>
</tr>
<tr>
<td>2-2-0</td>
<td>9</td>
<td>3</td>
<td>25</td>
<td>5-3-1</td>
<td>110</td>
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<tr>
<td>2-3-0</td>
<td>12</td>
<td>5</td>
<td>29</td>
<td>5-3-2</td>
<td>140</td>
</tr>
<tr>
<td>3-0-0</td>
<td>8</td>
<td>3</td>
<td>24</td>
<td>5-3-3</td>
<td>170</td>
</tr>
<tr>
<td>3-0-1</td>
<td>11</td>
<td>4</td>
<td>29</td>
<td>5-4-0</td>
<td>130</td>
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<td>3-1-0</td>
<td>11</td>
<td>4</td>
<td>29</td>
<td>5-4-1</td>
<td>170</td>
</tr>
<tr>
<td>3-1-1</td>
<td>14</td>
<td>6</td>
<td>35</td>
<td>5-4-2</td>
<td>220</td>
</tr>
<tr>
<td>3-2-0</td>
<td>14</td>
<td>6</td>
<td>35</td>
<td>5-4-3</td>
<td>280</td>
</tr>
<tr>
<td>3-2-1</td>
<td>17</td>
<td>7</td>
<td>40</td>
<td>5-4-4</td>
<td>350</td>
</tr>
<tr>
<td>4-0-0</td>
<td>13</td>
<td>5</td>
<td>38</td>
<td>5-5-0</td>
<td>240</td>
</tr>
<tr>
<td>4-0-1</td>
<td>17</td>
<td>7</td>
<td>45</td>
<td>5-5-1</td>
<td>300</td>
</tr>
<tr>
<td>4-1-0</td>
<td>17</td>
<td>7</td>
<td>46</td>
<td>5-5-2</td>
<td>500</td>
</tr>
<tr>
<td>4-1-1</td>
<td>21</td>
<td>9</td>
<td>55</td>
<td>5-5-3</td>
<td>900</td>
</tr>
<tr>
<td>4-1-2</td>
<td>26</td>
<td>12</td>
<td>63</td>
<td>5-5-4</td>
<td>1600</td>
</tr>
</tbody>
</table>

Table 3: MPN Index and 95% confidence limits for various combinations of positive results when five tubes are used per dilution
3.2 Short Term Nitrification Assay (STNA)

3.2.1 Materials

3.2.1.1 Phosphate buffer:

- Stock solutions:
  - dilute 3.48 g $K_2HPO_4$ / 100 ml (0.2 M)
  - dilute 2.72 g $KH_2PO_4$ / 100 ml (0.2 M)

Phosphate buffer (1): Add 4 ml of potassium monohydrogen phosphate ($K_2HPO_4$) and 1 ml of potassium dihydrogen phosphate ($KH_2PO_4$) stock solution per liter of distilled or deionized water (pH=7.1-7.4).

   Sterilize for 15 min at 121 °C.

Phosphate buffer (2): Dilute the stock solution two times and sterilize for 15 min at 121 °C.

   Air-saturate the phosphate buffer (2) by shaking in a partially filled bottle or by aeration with organic-free filtered air.

3.2.1.2 Ammonium sulfate [(NH₄)₂SO₄], 0.25 M (sterile).

3.2.1.3 Sodium chlorate (NaClO₃), 1.0 M (sterile).

3.2.1.4 Diazoting reagent: Dissolve 0.5 g of sulfanilamide in 100 ml of 2.4N HCl.

   Store the solution in a refrigerator.
3.2.1.5 Coupling reagent: Dissolve 0.3 g of N-(1-naphthyl)-ethylenediamine hydrochloride in 100 ml of 0.12N HCl. Store the solution in an amber bottle in a refrigerator.

3.2.1.6 Standard Nitrite Solution: dilute 0.274 g of NaNO₂ in 1000 ml. Solution contains 50 µg of NO₂-N per ml.

3.2.1.7 Nitrapyrin (2-Chloro-6-(Trichloromethyl)Pyridine), 0.05g diluted in 10 ml Etanol

3.2.2 NO₂-Calibration Graph

Dilute 20 ml of Standard Nitrite Solution to 1000 ml = 1 µg/ml

- Pipe 0 ml of 1 µg/ml solution and dilute to 100 ml volumetric flask = 0.00 µg/ml
- Pipe 1 ml of 1 µg/ml solution and dilute to 100 ml volumetric flask = 0.01 µg/ml
- Pipe 2 ml of 1 µg/ml solution and dilute to 100 ml volumetric flask = 0.02 µg/ml
- Pipe 4 ml of 1 µg/ml solution and dilute to 100 ml volumetric flask = 0.04 µg/ml
- Pipe 8 ml of 1 µg/ml solution and dilute to 100 ml volumetric flask = 0.08 µg/ml
- Pipe 16 ml of 1 µg/ml solution and dilute to 100 ml volumetric flask = 0.16 µg/ml
3.2.3 Calibration and Measurement

- Pipette an aliquot (usually 8 ml) of the solution into a 100 ml volumetric flask, add water to make the total amount about 90 ml.
- Add 2 ml diazotizing reagent, and mix the solution.
- After 5 min. add 2 ml of the coupling reagent. Mix the solution and allow it to stand for 20 minutes.
- Dilute the solution to volume (100 ml), mix it thoroughly and measure its color intensity at 520 nm against a reagent blank solution.
- Prepare the calibration graph.
- Calculate the NO$_2$-N concentration of unknown samples from a linear regression of the calibration curve.

3.2.4 Preparation of samples

- Centrifuge 50 ml of the mixed liquor for 20 minutes and remove the supernatant.
- Add phosphate buffer (1) to a volume of 50 ml, centrifuge again for 20 minutes.
- Remove the supernatant, replace by phosphate buffer (1).
- Shake well and dilute the washed solution with phosphate buffer (2) to the wished concentration (High, Medium and Low Dose Reactor 10 times, Nitrifying Bacteria Culture 100 times) place the solution in a 250-ml erlenmeyer flask.
3.2.5 Procedure

- Add 0.5 ml (for samples from High, Medium and Low Dose Reactor, Nitrifying Bacteria Culture: 2.0 ml) (NH₄)₂SO₄ solution

- Place flasks on a rotary shaker, and add 1.0 ml of chlorate solution (for samples from High, Medium and Low Dose Reactor, Nitrifying Bacteria Culture: 4.0 ml) per flask.

- Use parafilm to close the flask.

- Let shake for 30 minutes, then take a 15 ml aliquot.

- Centrifuge the 15 ml aliquot for approximately 5 minutes and take the supernatant for nitrite analysis.

- Collect samples in 30 minutes intervals, and determine nitrite concentration.
3.3 Respiration Activity Measurement

3.3.1 Materials

3.3.1.1 Phosphate buffer:

- Stock solutions:
  - dilute 3.48 g K$_2$HPO$_4$ / 100 ml (0.2 M)
  - dilute 2.72 g KH$_2$PO$_4$ / 100 ml (0.2 M)

Phosphate buffer (1): Add 4 ml of potassium monohydrogen phosphate (K$_2$HPO$_4$) and 1 ml of potassium dihydrogen phosphate (KH$_2$PO$_4$) stock solution per liter of distilled or deionized water (pH=7.1-7.4).

Sterilize for 15 min at 121 °C.

Phosphate buffer (2): Dilute the stock solution two times and sterilize for 15 min at 121 °C.

Air-saturate the phosphate buffer (2) by shaking in a partially filled bottle or by aeration with organic-free filtered air. The oxygen concentration has to be raised to more than 6 mg/l.

3.3.1.2 Ammonium sulfate [(NH$_4$)$_2$SO$_4$], 0.25 M (sterile).

3.3.1.3 Sodium chlorate (NaClO$_3$), 1.0 M (sterile).

3.3.1.4 Nitrapyrin (2-Chloro-6-(Trichloromethyl)Pyridine), 0.05g diluted in 10 ml Etanol

3.3.2 Preparation of samples

- Centrifuge 50 ml of the mixed liquor for 20 minutes and remove the supernatant.

- Add phosphate buffer (1) to a volume of 50 ml, centrifuge again for 20 minutes.
- Remove the supernatant, replace by phosphate buffer (1).

- Shake well and dilute the washed solution with phosphate buffer (2) to the wished concentration (High, Medium and Low Dose Reactor 10 times, Nitrifying Bacteria Culture 100 times) place 600ml of the solution in a flask.

3.3.3 Procedure

- Add 0.5 ml (for samples from High, Medium and Low Dose Reactor, Nitrifying Bacteria Culture: 2.0 ml) \((\text{NH}_4\text{)}_2\text{SO}_4\) solution per 100 ml to flask.

- Add 1.0 ml of chlorate solution (for samples from High, Medium and Low Dose Reactor, Nitrifying Bacteria Culture: 4.0 ml) per 100 ml.

- Mix the solution thoroughly. Place 300 ml of the solution in a BOD flask. Use two BOD flasks per sample.

- Add to one of the BOD flasks 0.1 ml of Nitrapyrin per 100 ml to inhibit the nitrification.

- Measure the dissolved oxygen concentration by using the BOD measurements.

- Determine the decrease of oxygen at appropriate intervals (every 10 to 15 minutes).

3.3.4 Results and Calculations

- Plot a graph showing the oxygen concentration verses the time and calculate the linear regression.

- Calculate the oxygen decrease during one hour
3.4 Standard Operation Procedure for Ammonia

3.4.1 Materials

Reagents: 1. 10N NaOH, dark bottle w/pipette dispenser

2. Stock NH₄Cl solution, 1.0 ml = 1.0 mg NH₃-N = 1000 mg/l-N

Apparatus: 1. Plastic beakers (50 ml)

2. Glass graduated cylinder (50 ml)

3. Magnetic stirrers

4. 10 ml pipettes, micro-pipette

3.4.2 Preparation of Standards:

1. 5 ml of stock solution dilute to 50 ml in 50 ml plastic beaker, mix solution with magnetic stirrer = 100 mg/l = 100 ppm

2. Pipet 5 ml 100 ppm solution and dilute to 50 ml in 50 ml plastic beaker = 10 ppm

3. Pipet 5 ml 10 ppm solution and dilute to 50 ml in 50 ml plastic beaker = 1 ppm

4. Pipet 5 ml 1 ppm solution and dilute to 50 ml in 50 ml plastic beaker = 0.1 ppm
3.4.3 Calibration and Measurement

1. Activate ORION 720A meter (with Ammonia probe)

2. Press 'calibrate', enter '4' for number of standards

3. Measure 45 ml of each standard in 50 ml plastic beaker

4. Add 1 ml 10N NaOH and measure probe immediately, mix solution with magnetic stirrer (start with 0.1 ppm standard).

5. Enter concentration when meter prompts

6. Repeat procedure for each standard (lowest to highest)

7. After last standard, the slope is generated by the meter. The slope must be between -56 and -60 mV, or the calibration procedure must be repeated

8. Once meter is calibrated, measure 45 ml sample and pour into a 50 ml beaker

9. Add 1 ml 10N NaOH and magnetic stirrer and insert probe in beaker immediately

10. Read concentration directly at beep as mg/l as NH$_3$-N
3.5 Standard Operation Procedure for TSS and MLTSS

3.5.1 Preparation of Filters
- Rinse filter with three consecutive doses of 20 ml distilled water
- Place filter in labeled planchette
- Place in muffle furnace (550 °C) for 20 min.
- Allow to cool and place in dessicator

3.5.2 Procedure
- Weigh filter and planchette in g (A)
- Measure volume of sample and pour through filter apparatus (V, ml)
- Place filter and planchette in 103 °C oven over-night
- Allow to cool in dessicator
- Weigh filter and planchette in g (B)
- Calculate TSS/ MLTSS (mg/l): (B-A)(1000)/V
- Save filter and planchette for VSS/MLVSS analysis
- Every 10 samples:
  Blank — same volume of distilled water passed through filter
  Duplicate — repeat procedure for same sample (may need to use ½ volume for sample and duplicate)
3.6 Standard Operation Procedure for VSS and MLVSS

3.6.1 Procedure

- Use sample volume passed through filter in ml from TSS (V)
- Use weigh of filter and planchette in g TSS (B)
- Place filter and planchette into muffle furnace 550 °C for 20 minutes
- Allow to cool, then place in dessicator
- Weigh filter and planchette in g (C)
- Calculate VSS (mg/l): \( \frac{(B-C)(1000)^2}{V} \)
4. Results and Discussion

4.1 Reactor Setup

The reactors were set up as described in the Master of Science thesis of Collins D. Lam (1997). In the present thesis the reactor set up is only briefly described.

For the present study the same bench-scale continuos-flow reactors were used. They are Plexiglas reactors with a total volume of 15.2 liters. Except for the front wall, which was made out of clear Plexiglas, all walls were black Plexiglas. The reactors were equipped with a clarifier baffle. Therefore, the volume of the aeration portion of the reactors was only 13.7 liters, whereas the clarifier portion was 1.5 liters.

The airflow to each reactor was regulated by rotometers, with a flowrate of 15 standard cubic feet per hour. The air is dispersed in the reactor through fine bubble ceramic diffuser stones. Two diffuser stones were used for each reactor. The average dissolved oxygen concentration was approximately 4 to 5 mg/l.
4.2 Sequencing Batch Reactor (SBR) to cultivate a Nitrifying Bacteria Culture (NBC)

To cultivate a nitrifying bacteria culture the clarifier baffle in one of the 15.2-liter reactors was removed. The initial seed was activated sludge from a local wastewater treatment plant. The reactor was aerated as described above.

After an aeration period of approximately 23 hours the air supply to the reactor was turned off and the sludge was allowed to settle for an hour. Afterwards approximately 10 liters of the supernatant was drained and the reactor refilled with tap water.

Then the air supply was turned on and 10 ml of concentrated Reactor-Feed (Feed compounds are listed in table 1) and 100 ml of ammonium chloride solution (100g/l) were added.

The pH was regulated with a pH controller to a pH between 6.5 to 7. When the pH fell below 6.5 a sodium hydroxide solution (2 to 3 Normal) was added automatically.

The ammonium concentration, as N-NH\textsubscript{4}, in the SBR was measured daily just before the settling process as well as after the refilling process.

After an acclimation period of approximately 30 days, the added ammonium was totally oxidized within 23 hours. The amount of daily-added ammonium chloride solution was then increased to 250 ml. After another 10 days, the ammonium was again totally reduced after 23 hours.

The steps are shown in the picture below (Picture 1).
Picture 1: Sequencing Batch Reactor (SBR)

1. Step: Air Supply on

2. Step: Air supply shut off, 1 hour settling time


4. Step: Refilling with tap water. Air supply on. Adding of Reactor feed and ammonium chloride solution
4.2.1 Sequencing Batch Reactor (SBR) to cultivate a Nitrifying Bacteria Culture (NBC) -

Results and Discussion

4.2.1.1 Ammonia Results

A plot of the ammonium concentration in the SBR is shown in Figure 2. Because of problems with the ammonium electrode during the first 30 days, only the second part, after increasing the amount of the added ammonium is shown.

![Ammonium Concentration in the NBC Reactor](image)

*Figure 2: Ammonium Concentration in the SBR, 31st - 44th Day*

<table>
<thead>
<tr>
<th>Day</th>
<th>31</th>
<th>32</th>
<th>33</th>
<th>34</th>
<th>35</th>
<th>36</th>
<th>37</th>
<th>38</th>
<th>39</th>
<th>40</th>
<th>41</th>
<th>42</th>
<th>43</th>
<th>44</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-NH$_4^+$ Concentration before Adding (I)</td>
<td>150</td>
<td>155</td>
<td>152</td>
<td>149</td>
<td>112</td>
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<td>20</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-NH$_4^+$ Concentration after Adding (I)</td>
<td>381</td>
<td>439</td>
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<td>401</td>
<td>415</td>
<td>400</td>
<td>416</td>
<td></td>
</tr>
</tbody>
</table>

(1) Adding of Ammonia and Reactor Feed Compounds
It can be assumed that the course of the ammonium concentration in the first 30 days was comparable. Measurement of the N-NH₄⁺-Concentration continued after the 44th day, but was no longer recorded.

4.2.1.2 MLSS and MLVSS Results

In the following plots, the MLSS and MLVSS results of the SBR reactor are shown. The samples were taken after the daily wasting and refilling process and before the ammonia chloride solution and the reactor feed were added. The samples were taken during the same time period as the steady-state nitrifier bioaugmentation tests.

**Figure 3: MLSS Concentration in the SBR during the bioaugmentation tests**

**Table 7: MLSS, Nitrifying Bacteria Culture**

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>11</th>
<th>15</th>
<th>18</th>
<th>22</th>
<th>25</th>
<th>29</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBC (mg/l)</td>
<td>4520</td>
<td>4750</td>
<td>4800</td>
<td>4820</td>
<td>4930</td>
<td>4780</td>
<td>4920</td>
<td>4770</td>
<td>4920</td>
<td>4580</td>
</tr>
</tbody>
</table>
The MLVSS concentration of the nitrifying bacteria culture was always between 4500 and 5000 mg/l, the MLVSS concentration was maintained between 4000 and 4500 mg/l.

It should be noted that the NBC was fed a mixed feed solution which allowed growth of a consortium of microorganisms. This means that only a fraction of the MLSS/MLVSS concentration was actually nitrifiers. The color of the NBC was initially light brown and gradually changed to a pinkish-brown color in concert with the increased ability to oxidize ammonium.
4.3 Steady-State Reactors for Nitrifier Bioaugmentation

Five reactors were set up as steady-state continuos-flow stirred-tank reactors (CTSTRs). The reactors were constantly fed with approximately 1200 times diluted Reactor-Feed (Feed compounds are listed in Table 1) at a flow rate of 18 ml/min and an ammonium chloride solution with a flow rate of 1 ml/min. These flow rates were chosen to obtain a Hydraulic Retention Time (HRT) of 12 hours in the aeration portion of the reactors. The Reactor-Feed was stored in a refrigerator and diluted automatically to reach a final COD concentration of approximately 500 mg/l.

To one of the five reactors, the ammonification control-reactor, no ammonium chloride was added, this reactor was operated to get an idea of the ammonium produced from the Reactor-Feed due to ammonification.

To the other four reactors, ammonium chloride was added to reach a steady N-NH$_4^+$ supply of 20 mg/l. Three reactors were inoculated daily with different amounts of cells derived from the NBC (bioaugmentation). The low-dose reactor received the least amount of bioaugmentation with settled cells from 20 ml of NBC. The medium-dose reactor received settled cells from 110 ml and the high-dose reactor received settled cells from 200 ml of the NBC.

The fourth reactor was not inoculated and was operated as a control reactor to compare the nitrification rate with and without bioaugmentation.

A three day Mean Cell Retention Time (MCRT) was chosen to ensure that full nitrification in the control reactor did not occur. This means that five liters of the reactor
volume had to be wasted from each reactor daily. Before the wasting process, the reactor
walls and the clarifier baffle were brushed to remove accumulated sludge. The mixed
liquor in the reactor was mixed thoroughly to waste a represent volume. Samples from
this mixed liquor were taken to carry out the Short Term Nitrification Assay (STNA),
Most Probable Number counting method (MPN), Mixed Liquor Suspended Solids
(MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS). All effluents from each
CFSTR was collected in 120 liter tanks.

The N-NH₄⁺ concentration in influent and effluent of all reactors were monitored daily.
The soluble COD in influent and effluent, the TSS, MLSS, VSS and MLVSS were
analyzed two times a week.

The SBR reactor are shown in the pictures below (Picture 2 and 3).
Picture 2: Continuous Flow Reactor

Continuously:  
- Adding of NH₄Cl - Solution (1 ml/min)  
- Reactor Feed (18 ml/min)

Discontinuously:  
- Daily wasting of 5 liters  
- Daily adding of Nitrifying Bacteria Culture (Bioaugmentation)
Picture 3: Reactor Setup for Continuous Flow Reactors

- Ammonium Feed
- SBR Enrichment Culture
- NH₄⁺ Control Reactor
- Control Reactor
- Low Dose Reactor
- Medium Dose Reactor
- High Dose Reactor
- Reactor Feed
- Effluent
4.3.1 Steady-State Nitrifier Bioaugmentation - Results and Discussion

The steady-state nitrifier bioaugmentation tests were completed after approximately 30 days. For five more days samples were taken and analyzed.

The control reactor received no bioaugmentation. The low, medium and high dose reactor received bioaugmentation dosages, as described before.

The pH in the reactors were not automatically controlled because as no pumps were available. In the control, low, medium and high dose reactors the pH was always between 6.5 and 7.5 hence, an automatic control was not necessary.

In the high dose reactor the pH dropped to lower than 6. After the 24th day, automatic pH control of this reactor started.

The results of the steady-state bioaugmentation tests are shown in the following graphs.
4.3.1.1 Ammonia Results

The results for ammonia during the bioaugmentation in the low, medium and high dose reactors are shown in the following graph and table (Figure 5 and Table 9). To the control reactor no nitrifying bacteria culture was added.

![Ammonia-Nitrogen Concentration](image)

**Figure 5: Ammonia Nitrogen Concentration during steady-state bioaugmentation**

The ammonia-nitrogen feed to the reactors was 20 mg/l. The high ammonia-nitrogen concentration in the reactors is a result of the ammonia produced by microorganism from the peptone and yeast extract in the reactor feed. If there was no ammonia oxidation in the control reactor, the produced ammonia from the feed was 30-40 mg/l.

The ammonia-nitrogen concentration in the effluent from the high dose CFSTR reached steady-state after the 26th day. In this reactor, steady-state would probably have been reached earlier if the pH was controlled automatically from the beginning. The ammonia-nitrogen concentration at steady state was approximately 3 mg/l. The medium dose
CFSTR reached steady-state after the 29th day. The concentration of ammonia-nitrogen in the effluent was then between 30 and 35 mg/l.

The control and low dose CFSTRs ammonia-nitrogen concentration in the effluent was between 50 and 55 mg/l in the control and between 46 and 51 mg/l in the low dose reactor.

It can be assumed that in the control reactor through the daily wasting process and therefore, the MCRT of 3 days no ammonia oxidation took place.

Therefore, the ammonia concentration in the control reactor seems to be representative for the actual ammonia concentration in the CFSTRs. The oxidation rate in the low, medium and high dose reactor can be calculated in comparison with the ammonia concentration in the control reactor and the ammonia concentration in the effluent of those reactors.

The results are shown in Table 9.
To measure the ammonia production from the peptone a fifth CFSTR was used. To this ammoniaification control reactor no external ammonia and no biomass was added. The ammonia concentration in the effluent of this reactor is shown in Figure 6 and Table 9.

![Ammonia-Nitrogen in the effluent of the Ammoniaification Control-Reactor](image)

Figure 6: Ammonia-Nitrogen in the effluent of the Ammoniaification Control-Reactor during steady-state bioaugmentation tests

The ammonia nitrogen concentration in the effluent of the ammoniaification control-reactor was around 40 mg/l. It can be expected that the ammonia production in this reactor was higher than in the reactors in which a bacterial culture was present and ammonia was externally added. The reason for this is that the activated sludge culture would consume some of the peptone before it could be converted into ammonia.

Another reason is that through the different conditions in the ammonification control reactor (no adding of NBC, no ammonia feed) a biomass in the reactor grewed which is not really comparable with the biomass in the other reactors. Through the daily adding of NBC and the continous ammonia feed to the low medium and high dose reactor the
composition of microorganism is different and therefore their production of ammonia through the reactor feed compounds.

4.3.1.2 Soluble Chemical Oxygen Demand (sCOD) Results

The sCOD results for the influent and effluent of each CFSTR are shown in the following figure.

![sCOD (Influent and Effluent)](image)

Figure 7: sCOD (Influent and Effluent) during steady-state bioaugmentation tests

Table 10: sCOD Concentration (mg/l) during the Bioaugmentation Tests

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>11</th>
<th>15</th>
<th>18</th>
<th>22</th>
<th>25</th>
<th>29</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Reactor Effluent</td>
<td>69</td>
<td>43</td>
<td>22</td>
<td>163</td>
<td>76</td>
<td>139</td>
<td>63</td>
<td>80</td>
<td>86</td>
<td>123</td>
</tr>
<tr>
<td>Low Dose Reactor Effluent</td>
<td>49</td>
<td>50</td>
<td>48</td>
<td>55</td>
<td>60</td>
<td>67</td>
<td>56</td>
<td>95</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>Medium Dose Reactor Effluent</td>
<td>160</td>
<td>65</td>
<td>44</td>
<td>51</td>
<td>92</td>
<td>82</td>
<td>64</td>
<td>102</td>
<td>77</td>
<td>103</td>
</tr>
<tr>
<td>High Dose Reactor Effluent</td>
<td>36</td>
<td>43</td>
<td>37</td>
<td>52</td>
<td>83</td>
<td>65</td>
<td>45</td>
<td>75</td>
<td>49</td>
<td>71</td>
</tr>
<tr>
<td>COD Influent</td>
<td>624</td>
<td>614</td>
<td>508</td>
<td>594</td>
<td>608</td>
<td>581</td>
<td>610</td>
<td>640</td>
<td>581</td>
<td>583</td>
</tr>
</tbody>
</table>
The average, incoming wastewater sCOD to the reactor was 600 mg/l. All of the reactors performed well. The average sCOD effluent for the control reactor was 80 mg/l, for the low dose reactor 75 mg/l, for the medium dose reactor 70 mg/l and for the high dose reactor 50 mg/l.

4.3.1.3 MLSS and MLVSS Results

In the following figures the MLSS and MLVSS results are shown. The samples were mixed liquor samples, taken after brushing the reactor walls.

<table>
<thead>
<tr>
<th>MLSS Concentration Table</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>11</th>
<th>15</th>
<th>18</th>
<th>22</th>
<th>25</th>
<th>29</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Reactor</td>
<td>1320</td>
<td>680</td>
<td>735</td>
<td>500</td>
<td>645</td>
<td>680</td>
<td>990</td>
<td>420</td>
<td>395</td>
<td>290</td>
</tr>
<tr>
<td>Low Dose Reactor</td>
<td>1280</td>
<td>840</td>
<td>785</td>
<td>705</td>
<td>635</td>
<td>600</td>
<td>675</td>
<td>718</td>
<td>405</td>
<td></td>
</tr>
<tr>
<td>Medium Dose Reactor</td>
<td>1320</td>
<td>780</td>
<td>1150</td>
<td>825</td>
<td>755</td>
<td>600</td>
<td>390</td>
<td>385</td>
<td>687</td>
<td>685</td>
</tr>
<tr>
<td>High Dose Reactor</td>
<td>1300</td>
<td>755</td>
<td>880</td>
<td>935</td>
<td>990</td>
<td>925</td>
<td>885</td>
<td>1070</td>
<td>1000</td>
<td>765</td>
</tr>
</tbody>
</table>

Figure 8: MLSS during steady-state bioaugmentation tests
The high dose reactor had the highest average MLSS at 874 mg/l, the medium and low dose reactors had average concentrations of 758 mg/l and 736 mg/l, respectively. The average MLSS for the control reactor was found to be 637 mg/l.

The MLVSS results are similar to the MLSS results.
4.3.1.4 TSS and VSS Results

In the following figures the TSS and VSS results are shown. The samples are taken from the effluent collection tanks after mixing.

![TSS Effluent Graph](image)

**Figure 10**: TSS during steady-state bioaugmentation tests

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>11</th>
<th>15</th>
<th>18</th>
<th>22</th>
<th>25</th>
<th>29</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Reactor</td>
<td>43.5</td>
<td>10.7</td>
<td>21.4</td>
<td>15.8</td>
<td>27.0</td>
<td>33.2</td>
<td>87.0</td>
<td>113</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>Low Dose Reactor</td>
<td>10.5</td>
<td>20.3</td>
<td>50.0</td>
<td>6.4</td>
<td>4.7</td>
<td>80.0</td>
<td>57.8</td>
<td>49.0</td>
<td>60.0</td>
<td>99.0</td>
</tr>
<tr>
<td>Medium Dose Reactor</td>
<td>6.4</td>
<td>12.3</td>
<td>41.0</td>
<td>10.0</td>
<td>18.8</td>
<td>209</td>
<td>204</td>
<td>64.0</td>
<td>54.0</td>
<td>78.0</td>
</tr>
<tr>
<td>High Dose Reactor</td>
<td>10.8</td>
<td>8.0</td>
<td>9.8</td>
<td>18.4</td>
<td>36.0</td>
<td>24.0</td>
<td>34.0</td>
<td>26.0</td>
<td>51.0</td>
<td>57.0</td>
</tr>
</tbody>
</table>

Table 13: TSS Concentration (mg/l) during the Bioaugmentation Tests
These figures, unfortunately, seem to indicate that steady-state was not achieved and that instead a period of increasing instability was beginning. This could be due to the design of the reactor internal clarifiers which are imperfect at retaining solids.
4.4 Short Term Nitrification Assay (STNA)

4.4.1 STNA - Conditions as described by Belser and Schmidt (1994)

The assay conditions described by Belser and Schmidt (1994) are shown in Table 15:

<table>
<thead>
<tr>
<th>Added (NH₄)₂SO₄-Solution (0.25 M)</th>
<th>0.2 ml per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Added NaClO₃-Solution (1.0 M)</td>
<td>1.0 ml per 100 ml</td>
</tr>
<tr>
<td>Dilution of the initial sample</td>
<td>20 g soil per 90 ml phosphate buffer (total volume: 100 ml)</td>
</tr>
<tr>
<td>Measuring</td>
<td>four to five samples spaced at 1- to 2-h intervals</td>
</tr>
</tbody>
</table>

Table 15: STNA Conditions described by Belser and Schmidt (1994)

These conditions were not found to be optimal to reach the maximum activity in initial trials. The reason is that the Belser and Schmidt described conditions are for soil samples. The samples used in this thesis were wastewater samples from the reactors described earlier (Chapter 4.3).

The following figures and table (Figures 12 and 13, Table 16) show the activity of the nitrifying bacteria culture in the reactor samples. To 100 ml of the undiluted wastewater samples from the different CFSTRs, 0.2 ml ammonium solution and 1.0 ml chlorate solution were added.
STNA (Control, Low, Medium and High Dose Reactor), conditions as described by Belser and Schmidt (1994)

![Graph](image)

Figure 12: STNA (Control, Low, Medium and High Dose Reactor), Conditions as described by Belser and Schmidt (1994)

STNA (Nitrifying Bacteria Culture), Conditions as described by Belser and Schmidt (1994)

![Graph](image)

Figure 13: STNA (Nitrifying Bacteria Culture), Conditions as described by Belser and Schmidt (1994)
Table 16: STNA, Conditions as described by Belser and Schmidt (1994);
Nitrite-N Concentration in pmol/ml

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>0</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Dose Reactor</td>
<td>482.3</td>
<td>986.0</td>
<td>1036.0</td>
</tr>
<tr>
<td>Medium Dose Reactor</td>
<td>399.8</td>
<td>882.3</td>
<td>979.0</td>
</tr>
<tr>
<td>Low Dose Reactor</td>
<td>312.3</td>
<td>741.0</td>
<td>877.3</td>
</tr>
<tr>
<td>Control Reactor</td>
<td>272.3</td>
<td>726.0</td>
<td>814.8</td>
</tr>
<tr>
<td>Nitrifying Bacteria Culture</td>
<td>2040.0</td>
<td>13440.0</td>
<td>16540.0</td>
</tr>
</tbody>
</table>

The concentration of Nitrite-N in the test reactors was initially (Time = 0) between 280 (Control Reactor) and 480 pmol/ml (High Dose Reactor), approximately. The Nitrite-N concentration in the Nitrifying Bacteria Culture was 2040 pmol/ml) initially.

The ammonia concentration in the initial samples (before addition of the ammonium solution) was between 20 and 30 mg/l in the Control, Low, Medium and High Dose Reactors. The ammonia concentration in the Nitrifying Bacteria Culture was too low to measure and therefore assumed to be 0 mg/l.

4.4.2 STNA - Wash Process for Initial Sample

To obtain comparable STNA’s, initially the concentration of ammonia and nitrite should be the same. Therefore the initial samples were washed with phosphate buffer as described earlier (Chapter 3.2.4).

After the washing process the ammonia and nitrite concentration in all samples was zero.

The results of the STNA with washed initial samples are shown in the following figure and table (Figure 14, Table 17).
After the washing process, the concentration of Nitrite-N in the initial samples were too low to determine with the Colorimetric Method (Chapter 3.2.5) and was assumed to be 0 pmol/ml.
The STNA of the NBC is shown in the following figure and table (Figure 15 and Table 18):

![Figure 15: STNA (Nitrifying Bacteria Culture), Washed Sample Conditions as described by Belser and Schmidt (1994)](image)

**Table 18: STNA (Nitrifying Bacteria Culture), Washed Sample Conditions as described by Belser and Schmidt (1994)**

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>0</th>
<th>0.167</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrifying Bacteria Culture</td>
<td>0</td>
<td>7340</td>
<td>4890</td>
<td>1790</td>
</tr>
</tbody>
</table>

The increase of Nitrite-N in the first ten minutes was 7340 pmol/ml. After two hours the Nitrite-N concentration went down to 4890 and after 4 hours to 1790 pmol/ml. The reason for this behavior was probably an insufficient chlorate concentration. Chlorate is an inhibitor of the second step of nitrification, the reaction of nitrite to nitrate (Chapter 2). When inhibition of the second step (Equation 2) is not complete, nitrite is oxidized to nitrate and cannot be determined anymore. Therefore, the concentration of chlorate needed to be increased.
4.4.3 Chlorate-Concentration

The sodium chlorate concentration used in the STNA described by Belser and Schmidt was 0.01 M per ml or 10 mM per liter.

4.4.3.1 Chlorate-Concentration for the Nitrifying Bacteria Culture

For the Nitrifying Bacteria Culture (NBC) the Chlorate concentration has found to be too low. This is shown in Figure 15. The measured concentration of Nitrite-N during the STNA in this test initially increased very fast and went then decreased. The reason was assumed to be the less than complete inhibition of the oxidation of nitrite to nitrate.

To determine the optimal chlorate concentration for STNA tests of the NBC, the initial sample was diluted 50 times and the NH₄-solution was unmodified from that described by Belser and Schmidt (1994) and the chlorate concentration was varied. The different chlorate concentrations are shown in the following table (Table 19).

<table>
<thead>
<tr>
<th>NBC</th>
<th>Added Chlorate Solution in ml</th>
<th>Final Chlorate Concentration in mM per liter (in 50times diluted initial sample)</th>
<th>Chlorate Concentration in initial sample in mM per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBC 1</td>
<td>8</td>
<td>80</td>
<td>4000</td>
</tr>
<tr>
<td>NBC 2</td>
<td>4</td>
<td>40</td>
<td>2000</td>
</tr>
<tr>
<td>NBC 3</td>
<td>2</td>
<td>20</td>
<td>1000</td>
</tr>
<tr>
<td>NBC 4</td>
<td>1</td>
<td>10</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 19: STNA (Nitrifying Bacteria Culture), Chlorate Concentration

The results are shown in the following figure and table (Figure 16, Table 20). The results are already multiplied by 50 (to account for the dilution rate).
The highest activity could be measured in the sample NBC 1. This sample also had the best correlation coefficient ($R^2$) with 0.9941.

In the NBC samples 2, 3 and 4, the measured Nitrite-N concentration was lower and the slope less accurate than in NBC sample 1.

The reasons for the strange initial results (Figure 15) are assumed to be due to only partial inhibition of the second nitrification step through chlorate in the samples of NBC culture which contains a high concentration of nitrifying bacteria.
4.4.3.2 Chlorate-Concentration for the Control, Low, Medium and High Dose Reactor Samples

To determine the optimal chlorate concentration for the Control, Low, Medium and High Dose Reactors, the initial samples were diluted 10 times, the NH₄-solution was maintained as described by Belser and Schmidt (1994), and the chlorate concentration was varied. The different chlorate concentrations tested are shown in Table 21.

<table>
<thead>
<tr>
<th>Control, Low, Medium, High Dose Reactor Number</th>
<th>Added Chlorate Solution in ml</th>
<th>Final Chlorate Concentration in mM per liter (in 10 times diluted initial sample)</th>
<th>Chlorate Concentration in initial sample in mM per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>20</td>
<td>200</td>
</tr>
</tbody>
</table>

Table 21: STNA (Control, Low Medium and High Dose Reactor), Chlorate Concentration - Results

In the following graphs (Figure 17-20) and tables (Table 22-25) the results of the STNA are shown. The results are already multiplied by 10 (to account for the dilution rate).
**STNA (Control Reactor), Chlorate Concentration**

![Graph showing chlorate concentration over time for control reactor with linear equations and R² values for each control sample.]

**Table 22: STNA (Control Reactor), Chlorate Concentration - Results**

<table>
<thead>
<tr>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope</th>
<th>Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>75.9</td>
<td>R² = 0.9883 ± 0.888</td>
</tr>
<tr>
<td>Control 2</td>
<td>294.0</td>
<td>R² = 0.9983 ± 0.500</td>
</tr>
<tr>
<td>Control 3</td>
<td>93.2</td>
<td>R² = 0.9989 ± 0.103</td>
</tr>
</tbody>
</table>

**STNA (Low Dose Reactor), Chlorate Concentration**

![Graph showing chlorate concentration over time for low dose reactor with linear equations and R² values for each low dose sample.]

**Table 23: STNA (Low Dose Reactor, Chlorate Concentration - Results**

<table>
<thead>
<tr>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope</th>
<th>Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low 1</td>
<td>567.4</td>
<td>R² = 0.9986 ± 0.794</td>
</tr>
<tr>
<td>Low 2</td>
<td>823.7</td>
<td>R² = 0.9948 ± 4.283</td>
</tr>
<tr>
<td>Low 3</td>
<td>694.6</td>
<td>R² = 0.9913 ± 6.043</td>
</tr>
</tbody>
</table>
STNA (Medium Dose Reactor), Chlorate Concentration

$\text{Medium 1} \quad y = 1519x + 127 \\
R^2 = 0.9817 \\
\text{Medium 2} \quad y = 1770x + 93.333 \\
R^2 = 0.9926 \\
\text{Medium 3} \quad y = 1599.2x + 112.08 \\
R^2 = 0.9867$

Figure 19: STNA (Medium Dose Reactor), Chlorate Concentration

<table>
<thead>
<tr>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope</th>
<th>Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 1</td>
<td>1646.0</td>
<td>$R^2 = 0.9817$</td>
</tr>
<tr>
<td>Medium 2</td>
<td>1863.3</td>
<td>$R^2 = 0.9926$</td>
</tr>
<tr>
<td>Medium 3</td>
<td>1711.3</td>
<td>$R^2 = 0.9867$</td>
</tr>
</tbody>
</table>

Table 24: STNA (Medium Dose Reactor), Chlorate Concentration - Results

STNA (High Dose Reactor), Chlorate Concentration

$\text{High 1} \quad y = 1296.2x + 68.122 \\
R^2 = 0.9917 \\
\text{High 2} \quad y = 2024.2x + 126.67 \\
R^2 = 0.9811 \\
\text{High 3} \quad y = 1332.5x + 103.75 \\
R^2 = 0.983$

Figure 20: STNA (High Dose Reactor), Chlorate Concentration

<table>
<thead>
<tr>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope</th>
<th>Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High 1</td>
<td>1364.3</td>
<td>$R^2 = 0.983$</td>
</tr>
<tr>
<td>High 2</td>
<td>2150.9</td>
<td>$R^2 = 0.9811$</td>
</tr>
<tr>
<td>High 3</td>
<td>1364.3</td>
<td>$R^2 = 0.9917$</td>
</tr>
</tbody>
</table>

Table 25: STNA (High Dose Reactor, Chlorate Concentration - Results
The highest activity could always be measured in sample number 2. These 10 times diluted samples received a chlorate concentration of 10 mM per liter.

In samples 1 and 3, the measured Nitrite-N concentration was lower and the slope in most of the samples was less accurate than in sample number 2.

These results can be interpreted to mean that the low chlorate samples (Samples number 1) had insufficient chlorate to fully inhibit the second nitrification step (Equation 2) and the high chlorate samples (Samples number 2) had excess chlorate which also inhibited the first nitrification step (Equation 1).
The highest activity could be measured in the sample NBC 1. This sample had a slope of 0.9941.

In the NBC samples 2, 3 and 4 the measured Nitrite-N concentration was lower and the slope less accurate than in NBC sample 1.

The reason for these results can be assumed to be too low of an ammonium concentration to reach the maximum activity. It can be concluded that the optimal initial ammonium concentration is 10 mM for the NBC samples.
4.4.4.2 Ammonium-Concentration for the Control, Low, Medium and High Dose Reactor

To determine the optimal ammonium concentration for the Control, Low, Medium and High Dose Reactors the initial samples were diluted 10 times, the chlorate concentration was kept on the optimum (Chapter 4.4.3.2; 10 mM) and the ammonium concentration was varied. The different ammonium concentrations are shown in the following table (Table 28).

<table>
<thead>
<tr>
<th>Control, Low, Medium, High Dose Reactor Number:</th>
<th>Added Ammonium-Solution in ml</th>
<th>Final Ammonium Concentration in mM per liter (in 10 times diluted initial sample)</th>
<th>Ammonium Concentration in initial sample in mM per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>1.25</td>
<td>12.5</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>2.5</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 28: STNA (Control, Low Medium and High Dose Reactor), Ammonium Concentration

In the following figures (Figures 22-25) and tables (Table 29-32) the results of the STNA are shown.
**STNA (Control Reactor), Ammonium Concentration**

![Graph showing STNA (Control Reactor), Ammonium Concentration](image)

- Control 1: $y = 297.62x - 3.6$, $R^2 = 0.9983$
- Control 2: $y = 277.17x + 3.6$, $R^2 = 0.9989$
- Control 3: $y = 295.6x + 4.96$, $R^2 = 0.9931$

**Table 29: STNA (Control Reactor), Ammonium Concentration - Results**

<table>
<thead>
<tr>
<th></th>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>280.8</td>
<td>$R^2 = 0.9989$</td>
</tr>
<tr>
<td>Control 2</td>
<td>300.5</td>
<td>$R^2 = 0.9931$</td>
</tr>
<tr>
<td>Control 3</td>
<td>294.0</td>
<td>$R^2 = 0.9983$</td>
</tr>
</tbody>
</table>

**STNA (Low Dose Reactor), Ammonium Concentration**

![Graph showing STNA (Low Dose Reactor), Ammonium Concentration](image)

- Low 1: $y = 861.67x - 37.917$, $R^2 = 0.9948$
- Low 2: $y = 818.51x - 40.933$, $R^2 = 0.9921$
- Low 3: $y = 836.28x - 15.128$, $R^2 = 0.9988$

**Table 30: STNA (Low Dose Reactor), Ammonium Concentration - Results**

<table>
<thead>
<tr>
<th></th>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low 1</td>
<td>777.6</td>
<td>$R^2 = 0.9921$</td>
</tr>
<tr>
<td>Low 2</td>
<td>823.8</td>
<td>$R^2 = 0.9948$</td>
</tr>
<tr>
<td>Low 3</td>
<td>821.2</td>
<td>$R^2 = 0.9988$</td>
</tr>
</tbody>
</table>
STNA (Medium Dose Reactor), Ammonium Concentration

![Graph showing ammonium concentration over time for Medium 1, Medium 2, and Medium 3.](image)

Figure 24: STNA (Medium Dose Reactor), Ammonium Concentration

<table>
<thead>
<tr>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 1 1415.2</td>
<td>$R^2 = 0.9840$</td>
</tr>
<tr>
<td>Medium 2 1862.6</td>
<td>$R^2 = 0.9925$</td>
</tr>
<tr>
<td>Medium 3 1863.3</td>
<td>$R^2 = 0.9926$</td>
</tr>
</tbody>
</table>

Table 31: STNA (Medium Dose Reactor), Ammonium Concentration - Results

STNA (High Dose Reactor), Ammonium Concentration

![Graph showing ammonium concentration over time for High 1, High 2, and High 3.](image)

Figure 25: STNA (High Dose Reactor), Ammonium Concentration

<table>
<thead>
<tr>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>High 1 1697.0</td>
<td>$R^2 = 0.9780$</td>
</tr>
<tr>
<td>High 2 2150.9</td>
<td>$R^2 = 0.9811$</td>
</tr>
<tr>
<td>High 3 2146.6</td>
<td>$R^2 = 0.9814$</td>
</tr>
</tbody>
</table>

Table 32: STNA (High Dose Reactor), Ammonium Concentration - Results
The Control, Low and High Dose Reactors reached the highest activity with a final ammonium concentration of 1.25 mM per liter of the 10 times diluted initial sample. Doubling this amount of ammonia (to 2.5 mM) did not cause a higher production of nitrite. The Medium Dose Reactor reached the highest activity with an ammonium concentration of 2.5 mM per liter. The difference between the nitrite production in the Medium 2 (1.25 mM ammonium per liter) and Medium 3 (2.5 mM ammonium per liter) was so low (0.7 pmol/ml*h) that it can be ignored.

In the Medium Dose Reactor the difference between the best working sample (Medium 3) and the worst working sample (Medium 1) was 448.1 pmol/ml*h.

In the High Dose Reactor the difference between High 2 (best working sample) and High 1 (worst working sample) was 453.9 pmol/ml*h.

It can be concluded that the optimal initial ammonium concentration is 1.25 mM for the Control, Low, Medium and High Dose Reactor samples.
The time period of 1.5 hours seemed to be enough to obtain sufficient plots for a linear slope.

To determine whether the inhibition through chlorate was complete during the test period of 1.5 hours, nitrapyrin was added after 0.5 hours to the samples. Nitrapyrin is a specific inhibitor of the first step of the nitrification (Equation 1). In the presence of both chlorate and nitrapyrin the ammonium oxidation and the production of nitrite were stopped. If the inhibition of chlorate was completely effective, the nitrite production should be trapped between the two inhibitors and the nitrite concentration should remain unchanged.

Two tests were carried out to achieve this complete inhibition. First nitrapyrin was added at the beginning of the assay to see if the inhibition of the first step (Equation 1) was complete. Second, nitrapyrin was added after 0.5 hours to see if the chlorate inhibition was complete. The results are shown in the following figures and tables.
Figure 26: Addition of nitrapyrin at the beginning of the assay

Table 33: STNA (Nitrifying Bacteria Culture), Inhibition through Nitrapyrin, Addition at the beginning
Nitrite-N in pmol/ml

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBC 1</td>
<td>0</td>
<td>6100</td>
<td>11725</td>
<td>15225</td>
</tr>
<tr>
<td>NBC 2</td>
<td>0</td>
<td>1075</td>
<td>1225</td>
<td>1350</td>
</tr>
</tbody>
</table>

Figure 26 and Table 34 indicate that Nitrapyrin did not completely inhibit the oxidation of ammonium.

The nitrite concentration in the inhibited test sample (NBC 2) increased in the first 30 minutes much more than during the 60th and 90th minute.

Reasons therefore can be that it needed some time at the beginning of the assay before nitrapyrin was completely distributed in the system.

A higher nitrapyrin concentration (10 µg/ml) did not cause less nitrite production.
Figure 27: Addition of nitrapyrin after 0.5 hours

STNA (Nitrifying Bacteria Culture), Inhibition through Chlorate

![Graph showing nitrite-N concentration over time with NBC 1 and NBC 2 conditions](image)

Figure 27: STNA (Nitrifying Bacteria Culture), Inhibition through Chlorate, Addition after 0.5 hours.

NBC 1: No Nitrapyrin added
NBC 2: 5 µg/ml Nitrapyrin added after 0.5 hours.

Table 34: STNA (Nitrifying Bacteria Culture), Inhibition through Nitrapyrin, Addition after 0.5 hours

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBC 1</td>
<td>0</td>
<td>6100</td>
<td>11350</td>
<td>14350</td>
</tr>
<tr>
<td>NBC 2</td>
<td>0</td>
<td>6100</td>
<td>6200</td>
<td>6150</td>
</tr>
</tbody>
</table>

Nitrapyrin was added after 0.5 hours. The concentration of nitrite during the 30th and 90th minute was almost constant. The small increase of nitrite after 0.5 hours can be a result of the time needed before the inhibition is completed. The small decrease of nitrite after 1 hour probably indicates a less than completely inhibited second nitrification step through chlorate.
The increase of nitrite in the nitrapyrin inhibited sample was more than 100 pmol/ml after the first 0.5 hour and again after the first hour. This is relatively small compared with the nitrite produced without adding of nitrapyrin.

It should be noted that the initial NBC sample was already 100 times diluted. The measured difference was also only 1/100 of the amount shown in the table and graph. Because several tests were carried out to control the inhibition through nitrapyrin and chlorate, it can be assumed that neither the inhibition of the ammonium oxidation nor the inhibition of the nitrite oxidation was 100% complete.

However, because of the comparatively insignificant increase of nitrite in Graph 26 and the comparatively insignificant decrease of nitrite in Graph 27, the inhibitions through nitrapyrin and chlorate can be assumed to be complete for all practical purposes.

4.4.3.2 Control, Low, Medium and High Dose Reactor

To reach the maximum STNA activity in the Control, Low, Medium and High Dose Reactor samples the optimal chlorate concentration was found to be 100 mM per liter. The optimal ammonium concentration was found to be 12.5 mM per liter (Chapter 4.4.3 and 4.4.4).

For the STNA tests these samples were 10 times diluted.

The most important feature of the STNA plots is the linearity of the nitrite production over the time period of the assay (W.T. Smorczewski and E.L. Schmidt).
The time period of 1.5 hours seemed to be long enough to get sufficient plots for a linear slope.

To test the completeness of inhibition through chlorate, nitapyrin was added initially to the Control, Low, Medium and High Dose Reactors and the concentration of Nitrite-N was measured. The results are shown in the following figures and table (Figures 28 and 29, Table 35).

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Dose Reactor (+)</td>
<td>0</td>
<td>20.3</td>
<td>33.2</td>
<td>33.7</td>
</tr>
<tr>
<td>Control Dose Reactor</td>
<td>0</td>
<td>22.9</td>
<td>40.6</td>
<td>64.5</td>
</tr>
<tr>
<td>Low Dose Reactor (+)</td>
<td>0</td>
<td>30.5</td>
<td>50.6</td>
<td>53.9</td>
</tr>
<tr>
<td>Low Dose Reactor</td>
<td>0</td>
<td>38.9</td>
<td>108.3</td>
<td>147.9</td>
</tr>
<tr>
<td>Medium Dose Reactor (+)</td>
<td>0</td>
<td>85.4</td>
<td>96.3</td>
<td>90.6</td>
</tr>
<tr>
<td>Medium Dose Reactor</td>
<td>0</td>
<td>377.1</td>
<td>658.3</td>
<td>895.6</td>
</tr>
<tr>
<td>High Dose Reactor (+)</td>
<td>0</td>
<td>84.3</td>
<td>95.3</td>
<td>97.8</td>
</tr>
<tr>
<td>High Dose Reactor</td>
<td>0</td>
<td>691.7</td>
<td>941.7</td>
<td>1358.3</td>
</tr>
</tbody>
</table>

Table 35: STNA (Control, Low, Medium and High Dose Reactor), Inhibition through Nitrapyrin

![Graph](Figure 28: STNA (Control and Low Dose Reactor), Inhibition through Nitrapyrin)
Similar to the NBC the inhibition through Nitrapyrin was not 100% complete.

To test the inhibition through chlorate, nitrapyrin was added to the STNA samples from the Medium and High Dose Reactors after 1 hour. The results are shown in the following figure (Figure 30).
Table 36: STNA (Medium and High Dose Reactor), Inhibition through Chlorate

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium Dose Reactor</td>
<td>0</td>
<td>150</td>
<td>322.5</td>
<td>315.2</td>
<td>305.4</td>
</tr>
<tr>
<td>High Dose Reactor</td>
<td>0</td>
<td>200</td>
<td>360.1</td>
<td>351.2</td>
<td>341.8</td>
</tr>
</tbody>
</table>

After the addition of nitrapyrin to the Medium and High Dose Reactor samples, the concentration of Nitrite-N decreased slowly (Figure 30). This means the nitrite oxidation was not totally inhibited through chlorate. The results (Figure 29) show that the Nitrite-N production was not totally inhibited through nitrapyrin. It can be assumed that the inhibition through chlorate was even less effective, because even though the ammonium oxidation was not totally completed through nitrapyrin, nitrite was still oxidized after the 1 hour and the addition of nitrapyrin.

To calculate the loss or underestimation of the actual ammonia-oxidation potential, much more analysis and tests would have been necessary than were possible during the time of this project.

Because of the short time of the assay and the minor loss of Nitrite-N during this time the inhibitions were assumed to be complete and no further correcting calculations were made.
4.5 Short Term Nitrification Assay (STNA) - Results

4.5.1 Nitrifying Bacteria Culture

To determine the comparability of the STNA, several STNA were made from the Nitrifying Bacteria Culture on different days. The NBC was chosen because the number of nitrifiers can be assumed to be relatively constant. Through the daily wasting and refilling process, the conditions in the SBR reactor are relatively constant and the number of nitrifiers should not vary as much as in the other reactors (CFSTRs), in which the nitrifying bacteria culture was added daily and partly washed out through the effluent.

The NBC samples were mixed liquor samples after the water changing process and before the addition of the reactor feed and the ammonium solution. The samples were prepared as described in Chapter 3.2.4. To reach the maximum activity 4 ml Chlorate solution (final concentration 4000 mM) and 2 ml Ammonium solution (final concentration 500 mM) were added to the 100 times diluted NBC sample. The results are shown in the following figure and table (Figure 31, Table 37).
STNA, Nitrifying Bacteria Culture

The Nitrite-N production of the NBC during the chosen time period varied between 10679 pmol/ml*h (6th day) and 13740 pmol/ml*h (1st day). The average Nitrite production rate for these four tests was 11906 pmol/ml*h. The relative percent different from the average value for each measurement was 15.4, 0.3, 10.3, and 5.4 %.

The reasons for these slight variations can be that the phases in which the samples were taken were different. The initial sample in the 100 times diluted solution is not totally representative, not necessarily the same amount of cells have to be active at the time of the assay and that even under the equal conditions in the SBR the concentration of nitrifiers varied slightly.

The results of the compared STNAs for the NBC did not vary that much (no more than 15 %) and they seem to be comparable.

<table>
<thead>
<tr>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 13740</td>
<td>0.9660</td>
</tr>
<tr>
<td>Day 5 11940</td>
<td>0.9492</td>
</tr>
<tr>
<td>Day 6 10679</td>
<td>0.9863</td>
</tr>
<tr>
<td>Day 11 11265</td>
<td>0.9947</td>
</tr>
</tbody>
</table>

Table 37: STNA, Nitrifying Bacteria Culture
4.5.2 High Dose Reactor

Different samples from the High Dose Reactor were taken during the nitrifying bioaugmentation test described in Chapter 4.2 and the activity was measured. The results are shown in the following figure and table (Figure 32 and Table 26).

![Graph showing nitrite-N production over time](image)

**Figure 32: STNA, High Dose Reactor**

<table>
<thead>
<tr>
<th>Day</th>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope R²</th>
<th>Ammonium Oxidation in the Reactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 16</td>
<td>294.05</td>
<td>R² = 0.9983</td>
<td>44.4 %</td>
</tr>
<tr>
<td>Day 17</td>
<td>534.17</td>
<td>R² = 0.9799</td>
<td>44.6 %</td>
</tr>
<tr>
<td>Day 23</td>
<td>685.25</td>
<td>R² = 0.9368</td>
<td>47.3 %</td>
</tr>
<tr>
<td>Day 28</td>
<td>1287.7</td>
<td>R² = 0.9677</td>
<td>94.7 %</td>
</tr>
</tbody>
</table>

Table 38: STNA, High Dose Reactor

It is obvious that the activity of the samples increased between the 16th and 28th day. The high dose reactor reached steady state (in terms of complete ammonia oxidation) after the 26th day (Chapter 4.2.1.2). It can be assumed that a Nitrite-N production through nitrifiers of 1287.7 pmol/ml*h is necessary to oxidize an ammonium concentration between 50 and 55 mg/l in the influent totally. Before the 24th day the pH in the High Dose Reactor was not controlled automatically (Chapter 4.2.1). The pH dropped during this time to less than
6. The STNA was always run at a pH of approximately 7. Therefore, the STNA was the measured activity of the nitrifiers at a pH of around 7. Because the pH in the CFSTR was not optimal, the activity may have been lower and the ammonium oxidation did not reach the same maximum rate observed in the STNA.

4.5.3 Medium Dose Reactor

Different samples were taken from the Medium Dose Reactor during the nitrifier bioaugmentation test described in Chapter 4.2 and the activity was measured. The results are shown in the following figure and table (Figure 33 and Table 39).

The ammonia-nitrogen concentration in the effluent of the medium dose reactor varied a lot during the bioaugmentation test period. This is shown in Chapter 4.2.1.2 and Figure 5. The measured activity seems to be roughly comparable with the ammonium oxidation.
rate in the CFSTR. For example on the days the ammonium oxidation in the was low (day 20 and 24), the measured activity (by STNA) was also low. On day 18 and day 30 the oxidation of ammonia in the Medium Dose Reactor was around 30 %, and the measured activity was around 460 pmol/ml*h. This was the highest reduction rate and the highest measured activity which could be reached in the Medium Dose Reactor during the bioaugmentation test period.

4.5.4 STNA, Control, Low, Medium and High Dose Reactor

A typical STNA from reactor samples of the Control, Low Medium and High Dose Reactors taken on the same day, are shown in Figure 34 and Table 40.

![STNA, Control, Low, Medium and High Dose Reactor (Day 15)](image)

Figure 34: STNA, Control, Low, Medium and High Dose Reactor

<table>
<thead>
<tr>
<th></th>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Slope</th>
<th>Ammonium Oxidation in the Reactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>151.74</td>
<td>$R^2 = 0.9921$</td>
<td>0 %</td>
</tr>
<tr>
<td>Low</td>
<td>254.86</td>
<td>$R^2 = 0.9999$</td>
<td>7.14 %</td>
</tr>
<tr>
<td>Medium</td>
<td>480.00</td>
<td>$R^2 = 0.9838$</td>
<td>38.9 %</td>
</tr>
<tr>
<td>High</td>
<td>593.33</td>
<td>$R^2 = 0.9757$</td>
<td>44.4 %</td>
</tr>
</tbody>
</table>

Table 40: STNA, Control, Low, Medium and High Dose Reactor
4.5.5 Error Calculation of the STNA

To calculate the error of the STNA method duplicate samples from the NBC, High, Medium and Low Dose Reactor were taken. Because at the time of 0 (hours), the Nitrite-N concentration was 0 (pmol/ml), the plots were forced to go through zero. The results are shown in the following figures and table (Figures 35 - 38 and Table 41).

![STNA, Error Calculation (Nitrifying Bacteria Culture)](image1)

Figure 35: STNA (NBC), Error Calculation

![STNA, Error Calculation (High Dose Reactor)](image2)

Figure 36: STNA (High Dose Reactor), Error Calculation
STNA, Error Calculation (Medium Dose Reactor)

![Graph showing two data sets for Medium 1 and Medium 2 with the equations and R² values.

Figure 37: STNA (Medium Dose Reactor), Error Calculation

STNA, Error Calculation (Low Dose Reactor)

![Graph showing two data sets for Low 1 and Low 2 with the equations and R² values.

Figure 38: STNA (Low Dose Reactor), Error Calculation

<table>
<thead>
<tr>
<th>Sample 1: Nitrite-N (pmol/ml*h)</th>
<th>Sample 2: Nitrite-N (pmol/ml*h)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBC</td>
<td>10264</td>
<td>10746</td>
</tr>
<tr>
<td>High Dose Reactor</td>
<td>1251.4</td>
<td>1269.3</td>
</tr>
<tr>
<td>Medium Dose Reactor</td>
<td>499.29</td>
<td>465.0</td>
</tr>
<tr>
<td>Low Dose Reactor</td>
<td>213.93</td>
<td>215.71</td>
</tr>
</tbody>
</table>

Table 41: STNA, Error Calculation
The highest difference was found to be between the two samples of the Medium Dose Reactor. The difference was 6.9%. The difference between the samples from the Nitrifying Bacteria Culture was 4.5%, from the High Dose Reactor 1.4% and from the Low Dose Reactor 0.8%.

It can be assumed, that the error of the linear regression and therefore of the Nitrite-N production is at the maximum 6.9%. This means, the results in these tests varied maximum by ± 6.9%. The average percent difference for the four previous graphs is 3.4%.

The standard deviation between the point pairs can be calculated by the difference (%) of the separate point pairs:

<table>
<thead>
<tr>
<th>Samples</th>
<th>Point Pairs (0.5 hours)</th>
<th>Difference (%)</th>
<th>Point Pairs (1.0 hours)</th>
<th>Difference (%)</th>
<th>Point Pairs (1.5 hours)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBC1</td>
<td>6100</td>
<td>0</td>
<td>11350</td>
<td>3.2</td>
<td>14350</td>
<td>5.7</td>
</tr>
<tr>
<td>NBC2</td>
<td>6100</td>
<td>11725</td>
<td>15225</td>
<td>4.3</td>
<td>1760</td>
<td>0.7</td>
</tr>
<tr>
<td>High 1</td>
<td>835</td>
<td>4.3</td>
<td>1322.5</td>
<td>1.9</td>
<td>1760</td>
<td>0.7</td>
</tr>
<tr>
<td>High 2</td>
<td>872.5</td>
<td>1347.5</td>
<td>1772.5</td>
<td>8.6</td>
<td>545</td>
<td>22.2</td>
</tr>
<tr>
<td>Medium 1</td>
<td>320</td>
<td>545</td>
<td>695</td>
<td>11.2</td>
<td>322.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Medium 2</td>
<td>292.5</td>
<td>423.75</td>
<td>705</td>
<td>0</td>
<td>222.5</td>
<td>310</td>
</tr>
</tbody>
</table>

Table 42: Calculation of the standard deviation

Standard Deviation: \( s = \sqrt{\frac{1}{(n-1)} \sum (x_i - \bar{x})^2} \) (Equation 5: Standard Deviation)

\[ x_{si} = 0 + 4.3 + 8.6 + 0 + 3.2 + 1.9 + 22.2 + 11.2 + 5.7 + 0.7 + 1.4 + 3.9 = 62.4 \text{ (%)} \]

\[ x_s = 5.2 \text{ (%)} \]

\[ s = 6.3 \text{ (%)} \]

Variance: \( \sigma^2 = s^2 \) (Equation 6: Variance)

\[ \sigma = 39.7 \]
4.5.6 Ammonium Oxidation Rate and Measured Activity (STNA)

In the following figure and table (Figure 39, Table 42) the relationship between the observed ammonium oxidation rate in the CFSTRs and the STNA measured ammonium oxidation rate are shown. All the STNA values from Chapter 4.5.3 and 4.5.4 are utilized.

**Ammonium Oxidation and Measured Activity (STNA method)**

![Graph showing the relationship between Nitrite-N and Ammonium Oxidation.](image)

\[
y = 0.0832x - 8.3243 \\
R^2 = 0.9545
\]

Figure 39: Ammonium Oxidation and Measured Activity (STNA)

Table 42: Ammonium Oxidation and Measured Activity (STNA):

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Nitrite-N (pmol/ml*h)</th>
<th>Ammonium Oxidation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Dose Reactor (Day 15)</td>
<td>593</td>
<td>44.4</td>
</tr>
<tr>
<td>High Dose Reactor (Day 16)</td>
<td>294.05(^{(1)})</td>
<td>44.6(^{(1)})</td>
</tr>
<tr>
<td>High Dose Reactor (Day 17)</td>
<td>534.17</td>
<td>44.6</td>
</tr>
<tr>
<td>High Dose Reactor (Day 23)</td>
<td>685.25</td>
<td>47.3</td>
</tr>
<tr>
<td>High Dose Reactor (Day 29)</td>
<td>1287.7</td>
<td>94.7</td>
</tr>
<tr>
<td>Medium Dose Reactor (Day 15)</td>
<td>480</td>
<td>38.9</td>
</tr>
<tr>
<td>Medium Dose Reactor (Day 18)</td>
<td>460.4</td>
<td>26.8</td>
</tr>
<tr>
<td>Medium Dose Reactor (Day 20)</td>
<td>205.3</td>
<td>8.5</td>
</tr>
<tr>
<td>Medium Dose Reactor (Day 24)</td>
<td>240.8</td>
<td>16.4</td>
</tr>
<tr>
<td>Medium Dose Reactor (Day 29)</td>
<td>486.57</td>
<td>31.1</td>
</tr>
<tr>
<td>Medium Dose Reactor (Day 30)</td>
<td>456.4</td>
<td>31.4</td>
</tr>
<tr>
<td>Low Dose Reactor (Day 15)</td>
<td>254.86</td>
<td>7.14</td>
</tr>
<tr>
<td>Low Dose Reactor (Day 30)</td>
<td>216</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{(1)}\)Value is not included in Figure 40
It is obvious that a correlation between the ammonium oxidation rate in the reactors and the with the STNA measured activity exists. Therefore, the activity could be calculated when the ammonium oxidation rate is known. Similarly, through the measurement of activity in a wastewater treatment plant using the rapid STNA test (1.5 hours), it can be determined whether bioaugmentation is necessary, thereby controlling the process.
4.6 Respiration Activity Measurement (RAM) - Results

The RAM method was carried out with the same initial samples as the STNA. The samples were taken from the reactor, treated as described before (Chapter 3.3.2) and the activity was measured through the decrease of oxygen. It was assumed that the conditions found to be suited for the STNA (Chapter 4.4) should also be correct for the RAM. This also had the benefit that the activity of the same initial samples and under the same working conditions were measured with both methods.

To determine the activity of the nitrifiers, two equal samples were prepared as described earlier (Chapter 3.3.2). To one of the samples nitrapyrin (Sample 2) was added to inhibit the nitrifyers. The decrease of oxygen in both samples were measured and the difference between sample 1 (no nitrapyrin) and 2 (with nitrapyrin) was assumed to be the oxygen decrease by the nitrifiers. The advantage of the RAM test is that measurements are made using just a DO meter which is very simple.

4.6.1 RAM - Nitrifying Bacteria Culture (NBC)

The samples of the NBC to carry out the RAM were 100 times diluted and treated as described earlier (Chapter 3.3.2). Nitrapyrin was added to NBC 2. The results are shown in Figure 40 and Table 44.
The amount of oxygen utilized by the nitrifiers, measured through the RAM method in the initial sample from the NBC was 2436 pmol/ ml*h.

4.6.2 RAM - High, Medium and Low Dose Reactor

The initial samples of the High, Medium and Low Dose Reactor to carry out the RAM were 10 times diluted and treated as described before (Chapter 3.3.2). Nitrpyrin was added to the samples High 2, Medium 2 and Low 2. The results are shown in the following figures (Figures 41- 43) and table (Table 45).
RAM, High Dose Reactor (10 times diluted)

![Graph showing dissolved oxygen levels over time for High Dose Reactor with linear regression equations and R² values.]

Figure 41: RAM, High Dose Reactor (10 times diluted)

RAM, Medium Dose Reactor (10 times diluted)

![Graph showing dissolved oxygen levels over time for Medium Dose Reactor with linear regression equations and R² values.]

Figure 42: RAM, Medium Dose Reactor (10 times diluted)
Figure 43: RAM, Low Dose Reactor (10 times diluted)

Table 45: RAM, High, Medium and Low Dose Reactor

<table>
<thead>
<tr>
<th></th>
<th>Decrease of Dissolved Oxygen (pmol/ml*h) in sample</th>
<th>Dissolved Oxygen (pmol/ml*h) in initial sample $^{(1)}$</th>
<th>Slope $R^2$ Variation</th>
<th>Ammonium Reduction in the Reactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>High 1</td>
<td>-66.138</td>
<td>-661.38</td>
<td>$R^2 = 0.9981$ +/- 1.26</td>
<td>94.6 %</td>
</tr>
<tr>
<td>High 2</td>
<td>-45.619</td>
<td>-456.19</td>
<td>$R^2 = 0.9987$ +/- 0.59</td>
<td>36.5 %</td>
</tr>
<tr>
<td>Medium 1</td>
<td>-20.447</td>
<td>-204.47</td>
<td>$R^2 = 0.9731$ +/- 5.50</td>
<td></td>
</tr>
<tr>
<td>Medium 2</td>
<td>-16.827</td>
<td>-168.27</td>
<td>$R^2 = 0.9900$ +/- 1.68</td>
<td></td>
</tr>
<tr>
<td>Low 1</td>
<td>-18.114</td>
<td>-181.14</td>
<td>$R^2 = 0.9875$ +/- 2.26</td>
<td>3.85 %</td>
</tr>
<tr>
<td>Low 2</td>
<td>-16.271</td>
<td>-162.71</td>
<td>$R^2 = 0.9935$ +/- 1.06</td>
<td></td>
</tr>
</tbody>
</table>

(1) Dissolved Oxygen in initial sample = Decrease of Dissolved Oxygen in sample * 10 (dilution rate)

The amount of oxygen utilized by the nitrifiers, measured through the RAM method in the sample from the High Dose Reactor was 205 pmol/ml*h, in the sample from the Medium Dose Reactor was 36 pmol/ml*h, and in the sample from the Low Dose Reactor was 18 pmol/ml*h.
It is obvious that not only the concentration of nitrifiers was higher in the High Dose Reactor but also the concentration of all oxygen consuming organisms was higher as well.

The sample Medium 1 consumed more oxygen than the sample Low 1 and the difference between the oxygen decrease in the samples from the Low Dose Reactor is smaller than in the samples from the Medium Dose Reactor.

The initial findings of the RAM indicate that the measured values of oxygen consumption rate parallel the nitrifier dosing rate. Due to the constraints additional testing of the RAM method was not possible. Because of the easy handling of the RAM measurements (DO meter) and the rapid available results it can be recommended that further tests to optimize the RAM method should be carried out.
4.7 Most Probable Number (MPN) Counts

The MPN counts were carried out using the procedure described earlier (Chapter 3.1). The MPN tubes were incubated for at least 10 weeks.

4.7.1 Consistency of the MPN Method

To determine whether the MPN method is consistent, three series of MPN tubes with the same initial sample were incubated. Therefore, a 50 ml sample of the NBC was taken, centrifuged and washed as described before (Chapter 3.1.3). Afterwards three 10 ml portions of the washed and thoroughly shaken sample were taken and diluted with phosphate buffer. The MPN tubes were incubated and analyzed weekly. The results are shown in the following figure and table (Figure 44, Table 46).

Figure 44: Consistency of the MPN Method

<table>
<thead>
<tr>
<th>Samples</th>
<th>E-08 (cells/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBC 1</td>
<td>1.1*10^8</td>
</tr>
<tr>
<td>NBC 2</td>
<td>0.9*10^8</td>
</tr>
<tr>
<td>NBC 3</td>
<td>1.3*10^8</td>
</tr>
</tbody>
</table>

Table 46: Consistency of the MPN Method
The number of nitrifiers counted in the triplicate series vary by about 30% between the highest and the lowest counted number. This means that in the lowest sample 30% less nitrifiers could be counted compared with the highest sample. One potential reason for this could be the cells were not sufficiently separated (not representative samples). For effective MPN counts, each cell has to be individually suspended and transferred efficiently during the dilution process. It can be assumed, that the dilution process was not effective enough and that cell units were transferred instead of individual cells. Therefore the highest counted number can assumed to be too high and the lowest counted number can be assumed to be too low.

4.7.2 MPN Counts during the Bioaugmentation Tests

MPN counts were carried out to compare the MPN method with the RAM and STNA methods. Therefore, the same initial samples were used. The cells were centrifuged and washed as described before (Chapter 3.1.3) and from the same washed samples all three methods were carried out.

The results of the MPN counts are shown in Table 47 and Figures 45 - 49.
The number of nitrifiers in the Control Reactor, counted with the MPN method, decreased from $1.6 \times 10^7$ to $5 \times 10^6$ (cells/ml) during the bioaugmentation test. This is a reduction of 68.8% and probably a result of the daily wasting process. To the control reactor no NBC was added and it was assumed that through a MCRT of 3 days very little or no nitrification could take place in this reactor.
The number of nitrifiers in the Low Dose Reactor, counted with the MPN method alternated between $5 \times 10^6$ and $5.0 \times 10^7$ (cells/ml) during the bioaugmentation test. The highest number of nitrifiers were counted at the 14th day of the bioaugmentation test. The ammonium oxidation rate on the Low Dose Reactor on this day was 12.2% (Table 9).

The number of nitrifiers in the Medium Dose Reactor, counted with the MPN method alternated between $7.0 \times 10^5$ and $2.4 \times 10^7$ (cells/ml) during the bioaugmentation test. The
highest numbers of nitrifiers were counted at the beginning of the bioaugmentation test and on the 7th day. On these days the ammonium oxidation rate in the Medium Dose Reactor was 4.8 and 11.9 % (Table 9), respectively.

The number of nitrifiers in the High Dose Reactor, counted with the MPN counting method was much lower than those for the Control, Low and Medium Dose Reactors. This is surprising because the High Dose Reactor received the highest daily dose of NBC during the bioaugmentation test and achieved the highest ammonium oxidation rate (almost 95 %).
4.7.3 MPN Counts - Results and Discussion

Through the test series, described and shown in Chapter 4.7.1 it is obviously, that care has to be taken in comparing of the MPN methods. Through the washing and dilution process, cell units can be transferred instead of individual cells. These cell units lead to different numbers of nitrifiers in the incubation tubes and probably to errors in the evaluation.

In Chapter 4.7.2 the results of the MPN counts during the bioaugmentation tests are shown. In Figure 50 (below) the results of the MPN counts of the Control, Low, Medium and High Dose Reactor are shown.

![MPN-Counts, Control, High, Medium and Low Dose Reactor](image)

It is obvious, that the Control, Low and Medium Dose Reactor higher numbers of nitrifiers could nearly always be determined than in the High Dose Reactor. This is surprising because the High Dose Reactor received the highest amount of NBC and reached a ammonium oxidation rate of almost 95 %. It is possible that through reactor
individual conditions, the biomass in the High Dose Reactor and, therefore, the nitrifiers were not kept back as effective as in the other reactors. Even so, the ammonium oxidation in this reactor reached the highest rate.

The counted number of nitrifier in the Medium Dose Reactor was also lower than in the Control and Low Dose Reactor. And yet the ammonium oxidation rate was also higher in the Medium Dose Reactor.

The number of nitrifiers in the Control Reactor was relatively high. This is surprising because through the daily wasting process only very little ammonia oxidation should take place in this reactor. The counted number of nitrifiers was high, the ammonium concentration measured in the effluent was high and therefore the oxidation rate low. The STNA measured activity in the Control Reactor was also lower (Chapter 4.5.4) than in all other reactors.

It seems that the activity (ammonium oxidation rate) of the nitrifier species in the NBC is much greater that that of the “native” species in the Control Reactor. This could explain the above results. It could be that the control culture contains large numbers of nitrifiers with very low ammonia oxidation rates because of the unfavorable conditions. Similarly, the NBC conditions could have selected a species with much higher oxidation rate capabilities.
4.8 Short Term Nitrification Assay (STNA) and Respiration Activity Measurement (RAM) by Comparison

To compare the STNA method with the RAM method, mixed liquor samples from the Nitrifying Bacteria Culture (NBC) and the different reactors were taken and treated as described earlier (Chapter 3.2 and Chapter 3.3). The same initial samples were used to carry out both tests. Therefore, at least a volume of 800 ml of the diluted initial samples were prepared.
4.8.1. Nitrifier Bacteria Culture

In the following figures and tables (Figures 51 and 52, Table 48 - 49) the STNA and RAM of the Nitrifying Bacteria culture are shown.

![Graph showing STNA (Nitrifying Bacteria Culture), RAM and STNA by Comparison](image)

**Figure 51: STNA (Nitrifying Bacteria Culture), RAM and STNA by Comparison**

NBC 1 and NBC 2 were similar samples and worked under the same conditions.

**Table 48: STNA (Nitrifying Bacteria Culture), RAM and STNA by Comparison**

<table>
<thead>
<tr>
<th>Nitrite-N Concentration in pmol/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hours)</td>
</tr>
<tr>
<td>NBC 1</td>
</tr>
<tr>
<td>NBC 2</td>
</tr>
</tbody>
</table>
Figure 52: RAM (Nitrifying Bacteria Culture, 100 times diluted), RAM and STNA by Comparison

NBC 3: same conditions as described before
NBC 4: 0.1 ml of nitrapyrin solution was additional added to stop the ammonia oxidation.

Table 49: RAM (Nitrifying Bacteria Culture), RAM and STNA by Comparison

<table>
<thead>
<tr>
<th>Dissolved Oxygen Concentration in pmol/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hour)</td>
</tr>
<tr>
<td>NBC 3</td>
</tr>
<tr>
<td>NBC 4</td>
</tr>
</tbody>
</table>

To compare the STNA with the RAM method the theoretical nitrite production and the theoretical number of nitrifiers in the initial samples were calculated. The results are shown in the following table (Table 50).
<table>
<thead>
<tr>
<th></th>
<th>Nitrite Production (pmol/ml)</th>
<th>Oxygen Decrease (pmol/ml) in initial sample</th>
<th>Oxygen Decrease through Nitrifier (pmol/ml)</th>
<th>Number of Nitrifier (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STNA NBC 1 and NBC 2</td>
<td>34861.7 (^{(1)})</td>
<td>-</td>
<td>23241.1 (^{(3)})</td>
<td>1.5*10^6 (^{(4)})</td>
</tr>
<tr>
<td>RAM, NBC 3</td>
<td>4141.5 (^{(2)})</td>
<td>5125</td>
<td>2761.0</td>
<td>1.8*10^5 (^{(4)})</td>
</tr>
<tr>
<td>RAM, NBC 4</td>
<td>2364</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 50: Nitrifying Bacteria Culture, RAM and STNA by Comparison

\(^{(1)}\) Nitrite-N (pmol/l) * 3.29 = Nitrite (pmol/l)  
\(^{(2)}\) Theoretical: 1 mol Nitrite per 1.5 mol O_2  
\(^{(3)}\) Theoretical: 1.5 mol O_2 per mol Nitrite  
\(^{(4)}\) Theoretical: 0.023 pmol nitrite per cell per hour

In comparison to the STNA, the RAM method underestimated the nitrite production and the number of nitrifiers by around 88%.

### 4.8.2. High, Medium and Low Dose Reactor

In the following figures and tables (Figures 53 - 58, Tables 51 - 56) the STNA and RAM of the High, Medium and Low Dose Reactors are shown.
4.8.2.1 High Dose Reactor

High 1 and High 2 were similar samples and worked under the same conditions.

Table 51: STNA (High Dose Reactor), RAM and STNA by Comparison

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>High 1</td>
<td>0</td>
<td>835</td>
<td>1322.5</td>
<td>1760</td>
</tr>
<tr>
<td>High 2</td>
<td>0</td>
<td>872.5</td>
<td>1347.5</td>
<td>1772.5</td>
</tr>
</tbody>
</table>
Table 52: RAM (High Dose Reactor), RAM and STNA by Comparison
Dissolved Oxygen Concentration in pmol/ml

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>0</th>
<th>0.167</th>
<th>0.33</th>
<th>0.5</th>
<th>0.667</th>
<th>0.833</th>
<th>1</th>
<th>1.167</th>
<th>1.33</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>High 3</td>
<td>220.6</td>
<td>212.5</td>
<td>205</td>
<td>198.1</td>
<td>189.4</td>
<td>183.8</td>
<td>175.6</td>
<td>168.4</td>
<td>161.3</td>
<td>152.8</td>
</tr>
<tr>
<td>High 4</td>
<td>220.6</td>
<td>210.3</td>
<td>200</td>
<td>188.1</td>
<td>177.2</td>
<td>164.7</td>
<td>154.1</td>
<td>142.5</td>
<td>131.9</td>
<td>119.3</td>
</tr>
</tbody>
</table>
4.7.2.2 Medium Dose Reactor

Medium 1 and Medium 2 were similar samples and worked under the same conditions.

Table 53: STNA (Medium Dose Reactor), RAM and STNA by Comparison

<table>
<thead>
<tr>
<th>Nitrite-N Concentration in pmol/ml</th>
<th>Time (hours)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 1</td>
<td></td>
<td>0</td>
<td>320</td>
<td>545</td>
<td>695</td>
</tr>
<tr>
<td>Medium 2</td>
<td></td>
<td>0</td>
<td>292.5</td>
<td>423.75</td>
<td>705</td>
</tr>
</tbody>
</table>
RAM (Medium Dose Reactor 10 times diluted), RAM and STNA by Comparison

![Graph showing dissolved oxygen concentration over time for Medium 3 and Medium 4]

\[
\text{Medium 3: } y = -20.447x + 230.65 \\
R^2 = 0.9731
\]

\[
\text{Medium 4: } y = -16.827x + 232.64 \\
R^2 = 0.99
\]

Figure 56: RAM (Medium Dose Reactor, 10 times diluted), RAM and STNA by Comparison

Table 54: RAM (Medium Dose Reactor), RAM and STNA by Comparison

Dissolved Oxygen Concentration in pmol/ml

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>0</th>
<th>0.25</th>
<th>0.5</th>
<th>0.75</th>
<th>1</th>
<th>1.25</th>
<th>1.5</th>
<th>1.75</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 3</td>
<td>233.8</td>
<td>227.2</td>
<td>219.5</td>
<td>212</td>
<td>207.5</td>
<td>203.4</td>
<td>200</td>
<td>195.9</td>
<td>192.5</td>
</tr>
<tr>
<td>Medium 4</td>
<td>233.8</td>
<td>229.3</td>
<td>224.2</td>
<td>219</td>
<td>214</td>
<td>210.6</td>
<td>206.8</td>
<td>204.5</td>
<td>200.1</td>
</tr>
</tbody>
</table>
4.7.2.3 Low Dose Reactor

Low 1 and Low 2 were similar samples and worked under the same conditions.

Table 55: STNA (Low Dose Reactor), RAM and STNA by Comparison
Nitrite-N Concentration in pmol/ml

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low 1</td>
<td>0</td>
<td>135</td>
<td>197.5</td>
<td>322.5</td>
</tr>
<tr>
<td>Low 2</td>
<td>0</td>
<td>135</td>
<td>222.5</td>
<td>310</td>
</tr>
</tbody>
</table>
### 4.8.2.4 High Medium and Low Dose Reactor - RAM and STNA by Comparison

<table>
<thead>
<tr>
<th></th>
<th>Nitrite Production (pmol/ml)</th>
<th>Oxygen Decrease (pmol/ml) in initial sample</th>
<th>Oxygen Decrease through Nitrifyer (pmol/ml)</th>
<th>Number of Nitrifyer (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STNA High 1 and 2</td>
<td>4203.8 (1)</td>
<td>-</td>
<td>6305.7 (1)</td>
<td>1.8*10^3 (4)</td>
</tr>
<tr>
<td>RAM, High 3</td>
<td>154.0 (2)</td>
<td>445.48</td>
<td>231.0</td>
<td>6.7*10^3 (4)</td>
</tr>
<tr>
<td>RAM, High 4</td>
<td>-</td>
<td>676.50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>STNA Medium 1 and Medium 2</td>
<td>1600.8 (1)</td>
<td>-</td>
<td>2401.2 (1)</td>
<td>7.0*10^4 (4)</td>
</tr>
<tr>
<td>RAM, Medium 3</td>
<td>24.1 (2)</td>
<td>204.47</td>
<td>36.2</td>
<td>1.0*10^3 (4)</td>
</tr>
<tr>
<td>RAM, Medium 4</td>
<td>-</td>
<td>168.27</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>STNA Low 1 and Low 2</td>
<td>712.3 (1)</td>
<td>-</td>
<td>1068 (1)</td>
<td>3.1*10^4 (4)</td>
</tr>
<tr>
<td>RAM, Low 3</td>
<td>12.3 (2)</td>
<td>162.7</td>
<td>18.4</td>
<td>5.3*10^2 (4)</td>
</tr>
<tr>
<td>RAM, Low 4</td>
<td>-</td>
<td>181.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 57: High, Medium and Low Dose Reactor, RAM and STNA by Comparison

(1) Nitrite-N (pmol/l) * 3.29 = Nitrite (pmol/l)

(2) Theoretical: 1 mol Nitrite per 1.5 mol O2

(3) Theoretical: 1.5 mol O2 per mol Nitrite

(4) Theoretical: 0.023 pmol nitrite per cell per hour

In comparison with the STNA the RAM method underestimated the nitrite production and the number of nitrifiers in the High Dose Reactor by 96 %, in the Medium Dose Reactor by 98.5 % and in the Low Dose Reactor by 98.2 %.
4.9 STNA, RAM and MPN by Comparison

To compare the three methods with each other the theoretical and counted numbers of nitrifiers were calculated. The results are shown in Table 58.

<table>
<thead>
<tr>
<th>Control Reactor (Day 32)</th>
<th>Low Dose Reactor (Day 30)</th>
<th>Medium Dose Reactor (Day 29)</th>
<th>High Dose Reactor (Day 28)</th>
<th>NBC (Day 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STNA</td>
<td>3.1*10⁴</td>
<td>1.0*10³</td>
<td>2.7*10⁵</td>
<td>1.5*10⁶</td>
</tr>
<tr>
<td>RAM</td>
<td>5.3*10²</td>
<td>1.0*10¹</td>
<td>6.7*10⁴</td>
<td>1.8*10⁵</td>
</tr>
<tr>
<td>MPN</td>
<td>5*10⁸</td>
<td>9.0*10⁴</td>
<td>5.0*10⁴</td>
<td>1.4*10⁸</td>
</tr>
</tbody>
</table>

Table 58: STNA, RAM and MPN by Comparison

The highest number of nitrifiers were always determined by the MPN method. In the Low Dose Reactor the STNA and the RAM underestimated the numbers of nitrifiers by about 29 000 times or 170 000 times, respectively. The RAM underestimated the STNA 580 times. The STNA and the RAM of the Medium Dose Reactor underestimated the MPN counts about 48 000 or 500 000 times, respectively and the RAM underestimated the STNA about 104 times.

The RAM of the High Dose Reactor underestimated the MPN about 21 000 times and the STNA 402 times. The STNA underestimated the MPN 520 times.

The STNA of the NBC underestimated the MPN counts 6 000 times. The RAM underestimated the MPN 50 000 times and the STNA 8.3 times.

The number of active cells, determined with the STNA and the RAM method was always much lower than the counted number by the MPN. The main reason could be, that through the activity measurement only the cells active at the time of the assay can be determined. To calculate the number of nitrifiers through the STNA and RAM it was assumed that the cells worked with their maximum activity and that the main nitrifiers
present were the nitrifiers of the strain Nitrosomonas sp. with an activity of 0.023 pmol/ml*h (Chapter 2). In the reactors a mixed culture of nitrifiers was present. The composition and, therefore, the average activity was unknown. As discussed earlier (Chapter 4.7.3) the activities of the different species may be very different.

It is quite possible that the number of nitrifiers in the Control and Low Dose Reactor was higher than in the Medium and Low Dose Reactor, but the composition and, therefore, the overall activity of the nitrifiers was smaller. This would explain the difference between the number of nitrifiers counted by the MPN method and the oxidation rates measured by STNA.

Surprising is still, that the numbers of nitrifiers in the Control and Low Dose Reactors were higher than in the High Dose Reactor. It can be assumed, that the mixed liquor of the High Dose Reactor contained less nitrifiers but that those nitrifiers had a higher activity. If this is the reason, the nitrifiers with the higher activity might have displaced the nitrifiers with lower activity, so that less nitrifiers had a higher activity.

The difference between the RAM and the STNA can be a result of the test conditions. It is possible that the RAM was not carried out under optimal conditions. Because of the lack of time no further tests to optimize the RAM conditions were carried out. Instead of this, the STNA conditions were assumed to be optimal for the RAM.

The STNA method seems to give the most accurate information about the proceedings in the reactors. A higher measured activity through the STNA almost always corresponded with a high actual oxidation rate in the reactor.
5. Conclusion and Recommendation

The reactor setup was successful and unproblematic. The High Dose Reactor reached steady-state after 26 days and the Medium Dose Reactor after 29 days. It can be assumed that the High Dose Reactor would have reached steady-state earlier, if the pH would have been controlled automatically earlier.

The reactor conditions were monitored by measuring the MLVSS, MLSS, VSS, TSS, sCOD and the ammonium concentration in the influent and effluent.

The activity and number of nitrifiers were determined with the MPN, STNA and RAM methods. To compare these methods, the theoretical number of nitrifiers (MPN) or active nitrifiers (STNA and RAM), respectively were calculated and values from the three methods compared.

The highest number of nitrifiers was determined by the MPN method. The STNA and the RAM highly underestimated this number. The main reason can be assumed to be the time period of the test. The MPN tubes had to be incubated for at least ten weeks. In this time all nitrifiers were assumed to be active and grown to a high number to determine them by their nitrite production.

The duration of the STNA and RAM tests were defined to be 1.5 hours. Therefore, only those cells which could be measured as active at the time of the assays were counted. A longer time period would probably yield higher numbers. The plot of the reduction of oxygen measured in the RAM tests were still linear after 1.5 hours, but longer STNA tests showed that nitrite production was not linear after 1.5 hours.
To calculate the statistical error of the STNA method, several STNAs with the same initial sample had to be carried out. In Chapter 4.7, STNAs with the same initial sample are shown. The course of the nitrite production from two test series with the same initial sample seems to be very close and therefore, the error seems to be small.

To calculate a statistical error for the STNA method more STNAs with the same initial sample should be carried out (at least five replicates).

The incomplete inhibition through chlorate was shown in Chapter 4.3.1.3. Through the incomplete inhibition of the second nitrification step (Chapter 2) nitrite was oxidized to nitrate. Therefore the measured nitrite production rate could not be determined perfectly. This might be the reason for the non-linear nitrite production after 1.5 hours. For all calculations, the nitrite production was assumed to be complete.

To determine the magnitude of the error of this assumption further tests are required.

The RAM method highly underestimated the STNA and MPN methods. The reason for this could be that the conditions, found to be optimal for the STNA, were not optimal for the RAM. Further tests are required to optimize the conditions of the RAM.

With the MPN counting method, the number of nitrifiers in the reactors could be determined. In the CFSTRs the lowest number of nitrifiers were found in the High Dose Reactor. This is surprising, because the High Dose Reactor received the highest amount of NBC. Surprising also, was that the numbers of nitrifiers in the Low Dose and Control Reactor were relatively high. By the MPN method, counted numbers did not seem to correlate with the ammonium oxidation rate in the reactors. The High Dose Reactor reached an ammonium oxidation rate of almost 93 % (day 28), but the number of nitrifiers, counted with the MPN method was 72 % lower than the number counted in the
Medium Dose Reactor with an ammonium oxidation rate of 31 % (day 29) or 84 % lower than the number of nitrifiers in the Control Reactor with an ammonium oxidation rate of 0 % (day 30).

These results can possibly be explained by the composition of the biomass and, therefore, of the nitrifiers in the reactors. It can be assumed, that the mixed liquor of the High Dose Reactor contained less nitrifiers, however these nitrifiers had a higher activity. If this is correct, the nitrifiers with the higher activity might have displaced the nitrifiers with lower activity, so that less nitrifiers but with a higher activity were present.

To determine and control the effectiveness of bioaugmentation and to control the nitrification by bioaugmentation in a wastewater treatment process, the STNA method seems to deliver useful results about the conditions and activity of the nitrifiers in-situ. The advance of the STNA method are, the results are available after a short time (1.5 hours) and that the activity measured with the STNA correlates with the ammonium oxidation rate in the reactor.
6.0 References


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