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An improved nitrifying enrichment to remove ammonium and nitrite from freshwater aquaria systems

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Abstract

The total ammonium nitrogen (TAN) concentration is often a key limiting water quality parameter in intensive aquaculture systems. Removing ammonia (NH_3) through biological activity is thus an important objective in aquaria and aquaculture system designs. In this study, the performance characteristics of a suspension of nitrifying cells (named ammonia binding inoculum liquid, ABIL) have been explored. This aqueous suspension contains a highly active, nitrifying microbial consortium that can be used to shorten the start-up period of a biofilter. Tests were performed in freshwater at lab scale (70 l, 20–24 °C). Results showed that the application of the consortium at a dose of 5 mg volatile suspended solids (VSS) l^{-1} assures a total removal of ammonium (NH_4^+) and nitrite species from 10 mg N l^{-1} to below the detection limit within a period of 4 days. Experimentally, at a substrate level of 10 mg TAN l^{-1}, a rate of biological ammonium and nitrite conversion of the order 0.3–0.5 g TAN g^{-1} VSS $^{-1}$ day^{-1} could be achieved by the consortium in the freshwater aquaria systems tested. Provided adequate aeration and dissolved oxygen (DO) levels of 6 mg l^{-1} or more, no important intermediary nitrite concentrations were found. Only a small amount of TAN was not recovered as nitrate and might have been lost through ammonia stripping. Pre-inoculating the nitrifiers in polyurethane (PU) sponges and installation of such sponges in the freshwater aquaria did not improve the effect compared to adding the consortium directly to the water. After 12 months preservation of the inoculum at 4 °C, no important decrease in ammonium

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removal activity and only a minor decrease in the nitrite removal rate of the consortium were noticed.

Keywords: Nitrification; Aquarium; Ammonium; Nitrite

1. Introduction

Fish stocking densities in aquaria and aquaculture systems are limited primarily by the dissolved oxygen (DO) concentrations and often, also by the ammonia (NH_3) concentrations especially in systems with high hydraulic residence times (Meade, 1985). Ammonia is formed as the principal end product from protein metabolism in fish. Fish expel NH_3 through their gills by branchial diffusion, and in freshwater fish, ammonium (NH_4^+) can also be branchially exchanged for a monovalent cation. Ammonia and ammonium originating from the gills comprise 60–90% of the total N excreted by fish (Forster and Goldstein, 1969; Rychly, 1980). Urea is also expelled through the gills and accounts for 9–27% of the soluble N excreted (Clark et al., 1985). Another source of ammonia in aquaria and aquaculture systems is microbial ammonification of organic nitrogen in uneaten feed residues and in fish feces. This particulate matter only accounts for 3.4–4.2% of the total nitrogenous waste load in tank systems (Clark et al., 1985).

Ammonia exists in water in two forms, i.e., as ionized ammonium ions (NH_4^+) and unionized ammonia. The two species together are indicated as total ammonium nitrogen (TAN). Unionized ammonia is the more toxic form. Levels above 0.1 mg NH_3-N l^{-1} are considered detrimental to fish (van Rijn et al., 1990) and in practical terms, fish should not be chronically exposed to NH_3-N levels of more than 50 μg N l^{-1} (Frances et al., 2000). For larval culture, even more stringent conditions are required: Guillen et al. (1994) reported high mortalities in groups of 1-day-old larvae of the Japanese croaker (*Nibea japonica*) exposed to 2.7 and 27 μg NH_3-N l^{-1}.

Nitrification is the process in which ammonium is oxidized to nitrate in a two-step process carried out by two different groups of chemolithoautotrophic bacteria. In the first step, ammonium-oxidizing bacteria (AOB) oxidize ammonium to nitrite, which is converted to nitrate by nitrite-oxidizing bacteria (NOB) in the second step (Focht and Verstraete, 1977). Therefore, nitrification is an important process in fish culture, both in home aquaria and commercial aquaculture systems, since it can convert toxic ammonia to nitrate which is considered relatively harmless to fish and can be kept at safe levels with regular water changes (Hargreaves, 1998).

Nitrite is formed in aquaria and aquaculture systems from the oxidation of ammonia by chemolithoautotrophic AOB, but it can also be formed as a consequence of denitrification activity in anoxic zones in the biofilter. The accumulation of nitrite can be toxic to fish and other aquatic organisms. Nitrite reacts with hemoglobin to form methemoglobin inhibiting the transport of oxygen resulting in methemoglobinemia or brown blood disease (Frances et al., 1998). In the literature, different concentrations of nitrite ranging from below 0.2 mg l^{-1} NO_2^--N (Blancheton, 2000) up to 12 mg l^{-1} NO_2^--N (Mazik et al., 1991; Chen and Lee, 1997) are being targeted as safe levels in

aquaculture systems. Different parameters like exogenous chloride concentrations, aquatic species, growth phase, exposure time, and pH affect the tolerance of the species for nitrite.

Because of the ability to remove the ammonium ion and nitrite from water, the AOB and NOB play an essential role in aquaria and aquaculture systems. Therefore, these systems usually provide a solid matrix, often called a biological filter, to promote the growth of AOB and NOB (Wheaton et al., 1994). However, AOB and especially NOB are slow-growing organisms (Bock and Koops, 1992) with doubling times for NOB ranging from 12 to 32 h (Ehrich et al., 1995). To shorten the time needed for the establishment of the AOB and NOB, commercial preparations are available to seed the aquatic environment. Past studies have generally shown these preparations to be poorly effective for unknown reasons (Timmermans and Gerard, 1990).

Recently, a new approach for the growth and maintenance of nitrifying cultures has resulted in the development of a product named ammonia binding inoculum liquid (ABIL). This is a liquid-mixed microbial enrichment. In this study, the nitrification performances of freshwater aquaria inoculated with this consortium were tested. It is shown that application of the consortium as inoculum removes the TAN in a reliable and reproducible way in a few days, with formation of low transient levels of nitrite.

2. Materials and methods

2.1. Growth of the consortium

The nitrifying suspension called ABIL was obtained from AVECOM (Belgium). It is cultivated in a 500-l reactor in fed-batch mode at LabMET. The nitrifying culture was obtained by gradual enrichment starting from natural surface water. The culture receives a daily load of 88 g N (58.7 g TAN day^{-1} as ammonium chloride and 29.3 g NO_2^--N day^{-1} as sodium nitrite) to selectively support the growth of the nitrifying bacteria. The feed also contains calcium carbonate as a carrier matrix, buffer, and carbon source. The pH of the reactor is further controlled at pH 7.0 by addition of NaOH. Temperature is kept at 22–24 °C. Compressed air is used to aerate the culture and maintain a dissolved oxygen level of 6.0 mg O_2 l^{-1} or higher. The cell density of the bacteria is maintained at 0.5 g volatile suspended solids (VSS) l^{-1} by harvesting the culture on a regular basis. The harvested cell suspension can be further concentrated to desirable cell densities by sedimentation or centrifugation.

2.2. Specific activity of the consortium

The specific nitrifying activity of the ABIL consortium at different TAN concentrations was determined as follows: 500-ml Erlenmeyer flasks filled with the consortium were placed on a rotary shaker (115 rpm) after the addition of a specific volume of an ammonium chloride solution (1 g TAN l^{-1}) to produce TAN concentrations in the range of 25–250 mg l^{-1}. To prevent the cells from settling and to assure oxygen saturation, each Erlenmeyer flask was equipped with a porous stone connected to an airblower. The nitrifying activity of

the consortium was determined by measuring the concentration of TAN in the Erlenmeyer flasks as a function of time. The concentration of TAN was determined with the distillation method using a 2200 Kjeltec Auto Distillation apparatus (Foss Tecator, Sweden) (Bremner and Keeney, 1965). The temperature of the room where the activity tests were done was kept constant at 28 °C. For every TAN concentration, a blank test was performed to assess the amount of nitrogen lost due to ammonia stripping. The blank test consisted of an Erlenmeyer flask filled with tap water and a specific volume of an ammonium chloride solution placed on the same rotary shaker and provided with a porous stone connected to an airblower.

2.3. Nitrification performances of the consortium in aquarium systems

The nitrification activity was tested in three 70-l freshwater tanks. Freshwater was prepared by mixing 75% tap water with 25% distilled water. The alkalinity of the resultant water was measured by titration of 100 ml of this water with 0.02 N HCl using a 719S Titrino automatic titrator (Metrohm, Switzerland) to pH 4.3 (Greenberg et al., 1992) and was found to be 4 meq l^{-1}.

Each tank was provided with two filter modules, each with an internal volume of ± 900 cm^3 and powered by an airlift system. These two filters were placed in two different corners of the tank. Forty percent of the filter volume was filled with a layer of polyester cotton covered with a layer of gravel (5–6-mm diameter) as a carrier material for the nitrifying bacteria. The air diffusers in the filters assured that the dissolved oxygen (DO) concentration in the tanks always remained above 6 mg O_2 l^{-1}. Additionally, the turbulence generated by the flow of water through the filters guaranteed proper mixing of the water in the tank and kept the temperature homogeneous. The temperature of the water was kept at 24 ± 1 °C by means of an electrical resistance of 100 W.

Each tank was dosed with ammonium chloride to give a TAN concentration of 10 mg l^{-1}. One hour after the tanks were inoculated with the consortium, the nitrification activity was monitored through daily analysis of the concentrations of TAN, NO_2^--N and NO_3^--N of samples taken in the free space of the tanks. TAN was determined using the direct photometric method with the Nessler reagent (Greenberg et al., 1992). NO_2^--N and NO_3^--N were determined with a Dionex Ion Chromatograph with an AS9HC column (Dionex, USA). The eluant was 9 mM Na_2CO_3 at a flow of 1 ml min^{-1}. A sample loop of 100 μl was used and detection was done with an electrochemical conductivity detector. A UVIKON 932 spectrophotometer (Kontron Instruments, Switzerland) was used for the analysis of TAN. Other parameters analyzed daily were the dissolved oxygen (DO) concentration, the temperature, the pH, and the alkalinity of the water. The DO was measured by means of a membrane covered amperometric electrode (COS 381 oxygen probe with a COM 381 meter) (Endress + Hauser, Belgium). The volatile suspended solids content of ABIL was determined by subtracting the ash content from the total suspended solids (TSS) content. Ash content and TSS content were determined gravimetrically (Greenberg et al., 1992).

A blank test was performed to investigate the amount of nitrogen lost through ammonia stripping. For the blank tests, the same set-up was used except that the consortium was not added to the tanks.

2.4. Effect of pre-inoculated polyurethane sponge

The effect of the addition of pre-inoculated polyurethane (PU) sponges to a corner filter was examined in three 70-l freshwater tanks. For this reason, small pieces of reticulated PU sponge (5–6 cm^3) with 10 pores cm^{-1} (Recticel, Belgium), were submerged in a cultivating reactor for 48 h. Afterwards, these PU sponges were placed in a 2-l container filled with tap water to remove excess sludge and nitrates and finally, approximately 16 cm^3 of pre-inoculated polyurethane sponge was installed into each corner filter at the beginning of the experiment. One hour after the addition of 10 mg TAN l^{-1}, the nitrification activity was determined through daily analysis of TAN, NO_2^--N, and NO_3^--N as described in Section 2.2. The amount of microbial cells present on the inoculated PU sponges was estimated by washing six pieces of pre-inoculated PU sponge and determining the VSS content of the washing solution.

A third type of experiment included both the uses of pre-inoculated polyurethane sponge and regular ABIL consortium to inoculate three 70-l freshwater tanks. Here, also the nitrification activity was determined as described under Section 2.2.

3. Results

3.1. Specific activity of the ABIL consortium

A series of batch-type experiments were carried out to determine the specific activity of the consortium at different TAN concentrations. For each concentration, the specific activity was calculated by subtracting the amount of TAN lost in a blank test from the TAN removal rate per gram VSS at that specific concentration. For the concentration range of 25–250 mg TAN l^{-1}, the specific activity was found to be in the range of 0.33 ± 0.12 g TAN g^{-1} VSS $^{-1}$ day^{-1} (mean $\pm$ standard deviation, $n = 3$). Over the course of these experiments, the pH decreased steadily from 8.5 to 7.5.

3.2. Activity tests in freshwater aquaria systems

3.2.1. Removal of 10 mg TAN l^{-1} in freshwater dosed at 5 mg VSS l^{-1} aquarium water

In the first experiment, the consortium was used to start-up three 70 l tanks. After inoculation with 170 ml ABIL at a sludge concentration of 1.9 g VSS l^{-1} (320 mg VSS per tank or 4.7 mg VSS l^{-1}), a first dose of 10 mg TAN l^{-1} was added to the water in the tanks. The mean values of the concentrations of inorganic nitrogenous compounds for the three tanks measured in time following the inoculation are shown in Fig. 1. After about 4 days, all TAN added to the tanks was oxidized, while no nitrite was detected. The TAN removal by the consortium occurred at an overall rate of 0.47 ± 0.003 g TAN g^{-1} VSS $^{-1}$ day^{-1} (mean $\pm$ standard deviation, $n = 3$).

A second dose of TAN was added after the complete removal of the first dose. This time, all TAN was removed over a period of 2 days. At the end of the experiment, nitrite concentrations of 2 mg NO_2^--N l^{-1} were measured.

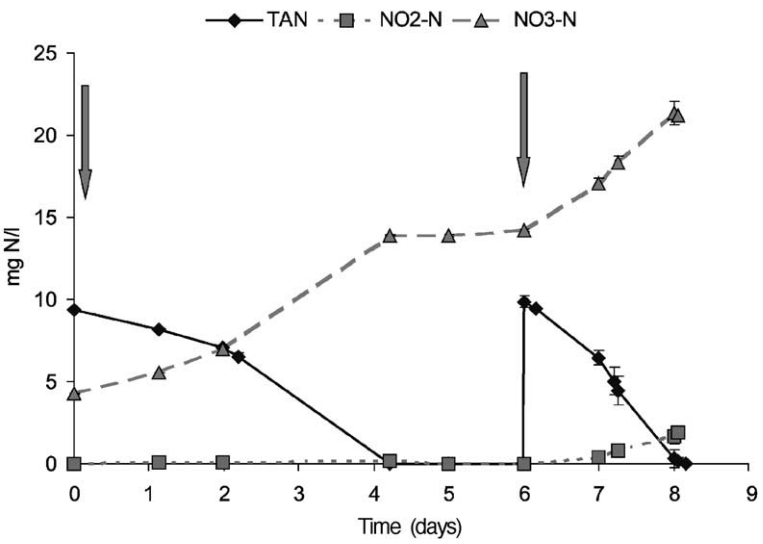

Fig. 1. Evolution of TAN, NO_2^--N, and NO_3^--N as a function of time, following the addition of two doses of TAN (indicated by arrow) to 70-l freshwater aquariums ($n=3$) inoculated with ABIL at 4.7 mg VSS l^{-1}. Data represent averages and bars indicate standard deviations.

No important imbalances occurred in the total nitrogen budget of the tanks after the first dose of TAN. During the removal of the second dose of TAN, approximately 0.7 mg N l^{-1} was not recovered. In a blank test, when adding 10 mg TAN l^{-1} without inoculating the tank with ABIL, 0.3 mg N l^{-1} was lost over a period of 4 days (data not shown).

3.2.2. Effect of pre-inoculated polyurethane sponge on the removal of 10 mg TAN l^{-1} in freshwater

Two types of experiments were performed in which the effect of the addition of pre-inoculated polyurethane sponges was investigated. In the first experiment, approximately 16 cm^3 of inoculated polyurethane sponge was installed in every filter in the tanks. This corresponded with 750 mg VSS per tank (10.7 mg VSS l^{-1}). Fig. 2 shows the mean values of concentration of inorganic nitrogenous compounds for three tanks measured in time after the addition of 10 mg TAN l^{-1}. TAN was removed at an overall rate of 0.12 ± 0.006 g TAN g^{-1} VSS^{-1} day^{-1}. All TAN added to the tanks was oxidized over a period of 9 days. Intermediate nitrite levels of less than 1 mg NO_2^--N l^{-1} were measured.

In the second type of experiment, both pre-inoculated polyurethane sponges and the regular consortium were added to the tanks. This resulted in a total ABIL dose of 1100 mg VSS per tank (15.7 mg VSS l^{-1}). Fig. 3 shows the mean values of concentration of inorganic nitrogenous compounds for the three tanks measured in time after the addition of 10 mg TAN l^{-1}. The TAN removal occurred at a rate of 0.11 ± 0.01 g TAN g^{-1} VSS^{-1} day^{-1} and 5 days were needed to oxidize all the TAN added. No net loss of nitrogen was observed in both experiments.

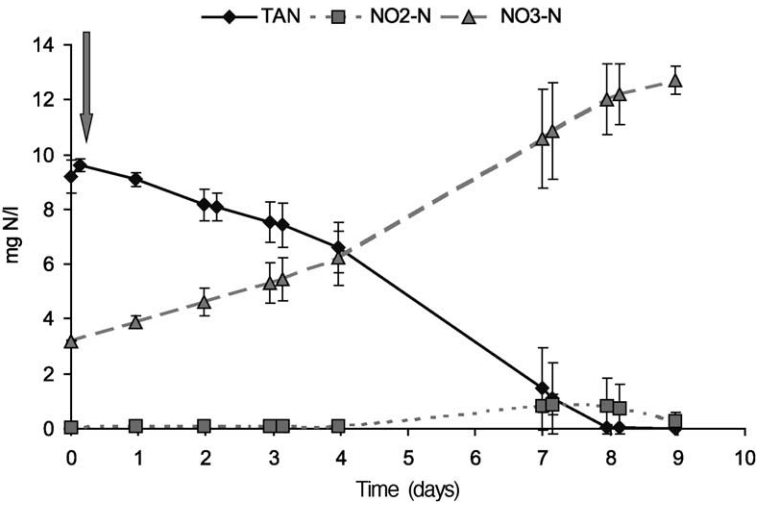

Fig. 2. Evolution of TAN, NO_2^--N, and NO_3^--N as a function of time, in 70-l freshwater aquariums ($n=3$) provided with pre-inoculated polyurethane sponge (with estimated ABIL amount of 750 mg VSS per aquarium, i.e., 10.7 mg VSS l^{-1}). Data represent averages and bars indicate standard deviations. Arrow indicates the addition of TAN.

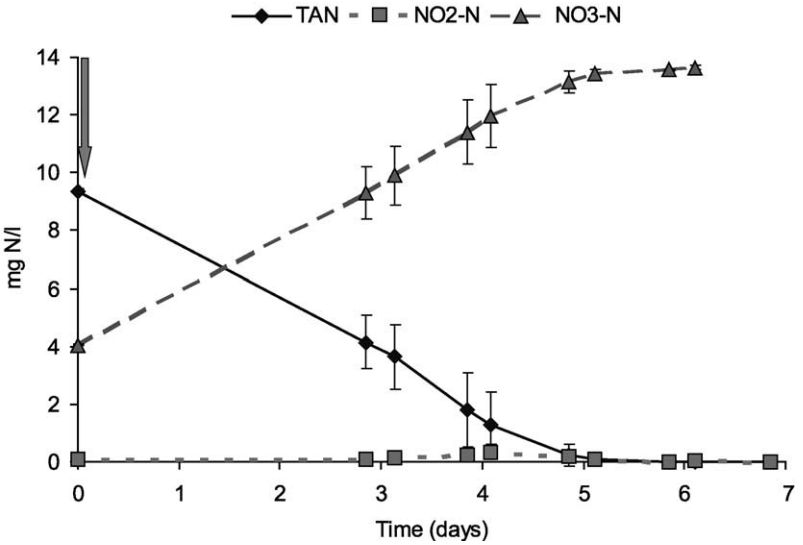

Fig. 3. Evolution of TAN, NO_2^--N, and NO_3^--N as a function of time, in 70-l freshwater aquariums ($n=3$) provided with pre-inoculated polyurethane sponge and inoculated with pure ABIL (with estimated ABIL amount of 1100 mg VSS per aquarium, i.e., 15.7 mg VSS l^{-1}). Data represent averages and bars indicate standard deviations. Arrow indicates the addition of TAN.

3.3. Preservation of the specific activity of the consortium upon storage

The activity of the ABIL nitrifying culture was examined after sealed storage of the nitrifying bacteria at 4 °C during 12 months.

Microscopic examination revealed that the ABIL flocs were for fresh and stored sludge alike, attached to the surplus $CaCO_3$ particles present in the medium. When tested after 12 months of storage under the conditions identical to those described in Section 2.3, the nitrification activity was still in the range of ± 0.5 g TAN g^{-1} VSS^{-1} day^{-1} ($n=1$). Yet, the test revealed that nitrite oxidation was slightly slower and that, when starting with such resting cells, nitrite could intermediary accumulate to 5–6 mg NO_2^--N l^{-1}.

4. Discussion

It has been reported that the start-up period of a biofilter in an aquaculture system takes from 28 to 60 days at temperatures between 21 and 26 °C (Carmignani and Bennett, 1977). Perfettini and Bianchi (1990) demonstrated that, without seeding the system with nitrifying bacteria, up to 40 days is required for the establishment of a nitrifying community. The time required to establish nitrification was shortened by about 30% of the time required without inoculation, when using frozen bacteria alone or combined with organic matter (food pellets) for the enrichment of seawater. As a result of the mineralization of the organic matter, ammonium was produced and became available for the growth of nitrifying bacteria before the introduction of the fish.

In contrast, the addition of the ABIL nitrifying culture clearly enhanced the oxidative conversion of TAN. Dosage of about 5 mg VSS l^{-1} water is sufficient to decrease the activation time to a few days as seen in the experiments (Fig. 1). It should be noted that all tests were performed in triplicate and demonstrated good reproducibility.

The use of pre-inoculated polyurethane sponge did not seem to improve the start-up period of the biofilters compared to the use of the ABIL suspension directly added to the aquarium water. Probably, the active cells were retained in the sponge pores and as such, less well supplied with essential nutrients such as oxygen and ammonium.

As can be seen from Figs. 1 and 2, the emergence of nitrites appears to be correlated with an increase in the TAN removal rate. This increase in AOB activity is probably a result of growth of the AOB, possibly resulting in an imbalance in numbers of AOB and NOB. A higher substrate concentration encountered by the AOB could be responsible for their higher growth rate. Traditionally, incomplete nitrification resulting in elevated nitrite levels has been attributed to inhibitory effects of parameters such as NH_3, high pH, low DO, and low temperature on NOB (Anthonisen et al., 1976; Randall and Buth, 1984; Huang et al., 1989; Hanaki et al., 1990; Balmelle et al., 1992; Yang and Alleman, 1992; Fdzpolanco et al., 1994). The situation described here, however, differs from the situations described by these authors. Indeed, most of the studies on the emergence of nitrites have been conducted on wastewater treatment plants where such extreme conditions are more likely to occur. During the experiments conducted for this study, the pH, temperature, DO, and TAN were all at optimal noninhibitory levels. The

inoculum appears to be sufficiently balanced in terms of AOB and NOB, as no important nitrite buildup could be detected at the start of the experiments.

A comparison of the specific activity of the ABIL consortium determined in batch experiments with values in literature for specific nitrifying organisms allows estimating the relative microbial concentration of the consortium. Neufeld et al. (1986) calculated the maximum substrate utilisation rate for axenic *Nitrosomonas* sp. to be 1.04 g TAN g^{-1} VSS^{-1} day^{-1}. The activity tests with the ABIL suspensions indicated substrate utilisation rates in the range of 0.3–0.5 g TAN g^{-1} VSS^{-1} day^{-1}, i.e., about 30–50% of the maximum specific rate reported for *Nitrosomonas* sp. value. Fluorescence in situ hybridisation analyses have revealed that the major active nitrifying organisms in the consortium are *Nitrosococcus*, *Nitrosomonas*, and *Nitrobacter* species (Wagner, 2000, personal communication). Clearly, the culture contains a diversity of nitrifiers and commensalic bacteria, which, by means of molecular analyses, need to be further characterised (Boon et al., 2000). The consortium has the specific advantage that the cells are attached to the carrier matrix, that it attains a considerable specific activity at relatively unfavourable levels (≤ 10 mg TAN l^{-1}), and that it can be produced and preserved in a reliable way.

These characteristics are of particular value for application in aquaculture systems. To the best of our knowledge, the availability of such an effective nitrifying consortium for reproducible inoculation of aquaria has not been reported before. This approach warrants further exploration in terms of microbial ecology and food chain interactions.

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