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NITRATE REMOVAL POTENTIAL OF DIFFERENT MICROBIAL CONSORTIA, FEASIBLE FOR WASTEWATER TREATMENT IN RAS

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Abstract
In Recirculating Aquaculture System (RAS) the water effluent from fish tanks is reused after cleansing mainly with respect to organic substances, nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and phosphate (PO_4^{-3}) ions. One of the most used and promising cleansing techniques in modern RAS is the biological treatment of wastewaters performed by denitrifying bacteria which can convert the NO_3^- to N_2. In this study we estimated the aerobic denitrifying performances of different microbial consortia isolated from various sources, feasible to be further used in RAS. The kinetics of NO_3^- consumption in either batch or discontinuous conditions, on synthetic culture media with acetate or ethanol as sole carbon source and nitrate as the main final electron acceptor, has been characterized. The experimental data showed that the selected microbial populations can clean water with efficiencies between 86% to100%, matching the requirements of international laws and making them suitable for employment in wastewater treatment in RAS.

Keywords: activated sludge, bacterial denitrification, microbial consortia, recirculating aquaculture system.

1. INTRODUCTION

Aquaculture is a fast growing industry, and it is expected that in the near future will significantly increase its contribution to the fish market (Goldburg and Naylor, 2005). For this to happen it is necessary to intensify their current production. But this comes with the cost of an increased discharge of waste compounds in the aquaculture effluents. The main aquaculture waste compounds that pollute the receiving natural waters are organic substances, nitrogen-containing compounds, and phosphates. At the present, Recirculating Aquaculture Systems (RAS) represents the solution that addresses these inconveniences. RAS is a modern technology in which spent water can be reused, therefore minimizing the waste output and increasing the recycling of water resources. The core of RAS is the biofiltration of spent water mainly with respect to organic substances, nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{-3}) (Crab et al., 2007; Dalsgaard et al., 2013). High concentrations of nitrogen-containing compounds in the receiving waters, mainly in the form of nitrate and ammonium, can lead to serious problems such as water eutrophication, being a potential threat to all aquatic organisms and of human health (Camargo et al., 2005; Addiscott and Benjamin, 2013). Therefore, limiting the nitrogen-containing compounds release into the environment had become the main concern nowadays.

Denitrification is defined as the microbially mediated reduction process of nitrate (NO_3) and nitrite (NO_2) to gaseous forms of nitrogen, principally nitrous oxide (N_2O) and nitrogen(N_2) (Skiba, 2008). Firstly, denitrification was considered a completely anaerobic process (Tiedje et al., 1982) but the

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discovery of the aerobic denitrification bacteria *Thiosphaera pantotropha* (Robertson and Kuenen, 1983) demonstrated that denitrification is not an oxygen limited process, occurring even at more than 80% air saturation (Robertson et al., 1989; Bell et al., 1990; Robertson and Kuenen, 1990). Aerobic denitrification has the advantage of being less costly and at times more efficient as compared to conventional anaerobic denitrification (Takaya et al., 2003), and its use could be the solution for a modern RAS design.
Therefore, the aim of the present study was to select and investigate the capacity of different denitrifying microbial consortia for high rate aerobic denitrification processes.

2. MATERIALS AND METHODS

Media and culture conditions for microbial consortia. Twelve microbial consortia were isolated from different sources (microbiota from fish farm biofilters and fish tanks waters (A1, A2, A3, A4, PBb, PBs), activated sludge from a municipal waste water treatment plant (Raja 3), an aquaria biofilter (AF, BOD) and three soil samples (S1, S2, S3)) and enriched in aerobic denitrifying bacteria using acetate as carbon source. Enrichment was performed according to literature (Takaya et al., 2003) by using a series of transfers onto screening media (SM) for heterotrophic denitrifying bacteria and maintained in our laboratory at 4^{o}C, on heterotrophic denitrification medium (DM) slants (g/L): sodium acetate, 4.72; $NaNO_3$, 1.62; $MgSO_4 \cdot 7H_2O$, 1; KH_2PO_4, 1.50; Na_2HPO_4, 0.42; NH_4Cl 0.6; casamino acids 5; trace element solution, 2.00 ml; agar, 20; pH 7.0-7.2.
Reduction of nitrate. To obtain rapid population expansion for denitrification assay, microbial consortia were revived by aerobic cultivation for 24h, at 30^{o}C in liquid DM medium. Cells were collected by centrifugation at 6500 rpm and 4^{o}C for 10 min. The obtained biomass was washed twice with water saline solution (0.9% NaCl), diluted with sterile water saline solution to an optical density (OD660) of 1 and inoculated to 1% (v/v) into experimental flasks with nitrate denitrification medium (NDM) (g/L): sodium acetate 5; $NaNO_3$, 0.37; $MgSO_4 \cdot 7H_2O$, 1; KH_2PO_4, 1; K_2HPO_4, 1; $CaCl_2 \cdot 2H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.05; trace element solution, 2.00 mL; pH 7.0-7.2. For denitrification assay, all the bacterial cultivations were conducted at 30^{o}C without shaking. During nitrate removal from NDM, microbial growth was monitored at OD660 and the kinetics of microbial growth was established. Time-dependent consumption of NO_3 was measured spectrophotometrically on Specord® 210 Plus (Analytik Jena) using the Spectroquant® Nitrate test kit (MerckMillipore) (method is analogous to DIN 38405-9). All the NO_3 concentrations were calculated based on a standard curve prepared with Nitrate IC-STD solution (MerckMillipore).
The influence of different C/N ratios. In experiments to test the effect of C/N ratio, the activated sludge from the municipal sewage treatment plant was used as source of denitrifying microorganisms, which were selectively cultivated in effluent water from the treatment plant enriched with ethanol as the sole carbon source, and the nitrate concentration was varied to yield different C/N ratios (5/1, 10/1, 250/1): 400 mg/L ethanol and 80 mg/L nitrate for the 5/1 ratio, 800 mg/L ethanol and 80 mg/L nitrate for the 10/1 ratio, and 20 g/L of ethanol and 80 mg/L of nitrate for the 250/1 ratio. Determination of nitrate concentration at the beginning of the experiment and after 24 hours was done with the kit Hach Lange LCK 340.
Statistical analysis. Data in this experiment was analysed by Microsoft Excel software. The denitrification rate formula is $(C_0 - C_n)/h$. C_0 is the initial concentration of NO_3. Cn is the final concentration of NO_3 at n hour. h is the time of microbial treatment. The nitrate removal efficiency formula is $(C_0 - C_n)/C_0 \times 100\%$. C_0 is the initial concentration and C_n is the final concentration of NO_3.

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3. RESULTS AND DISCUSSIONS

Denitrification characteristics of microbial consortia

Twelve distinct microbial populations were isolated and selected based on their ability to remove nitrate from a synthetic growth medium. After several successive passages on selective denitrification media (SM and DM), they were enriched in heterotrophic bacteria capable of aerobic denitrification. The growth and nitrate removal characteristics of the enriched microbial consortia were investigated in batch cultures, using a synthetic basic medium with sodium acetate (CH_3COONa) as sole carbon source and sodium nitrate ($NaNO_3$) as sole nitrogen source (i.e., NDM medium). The kinetics of NO_3 removal from the NDM medium during the aerobic growth of the enriched microbial populations was plotted in Figure 1a. All the microbial consortia tested exhibited high nitrate removal capacities, the concentrations of NO_3 decreasing significantly after the first 24 h of incubation, the bacterial populations tested being able to remove between 7.27 (4.76%) and 122.83 (63.42%) mg NO_3/L from the initial NO_3 concentration. The NO_3 removal rates varied between 0.3 and 5.12 mg NO_3/L/h, depending on the microbial consortia tested. As the microbial growth proceeded with the exponential growth phase, the removal of nitrate continued with different velocities, the microbial populations tested removing between 43.75 (28.64%) and 145.34 (90.59%) mg NO_3/L from the initial NO_3 concentration. The NO_3 removal rates varied between 0.9 and 3.03 mg NO_3/L/h during the exponential growth phase. The majority of the microbial consortia tested reached the stationary phase after 48 h of incubation, reaching their maximum OD660 values (between 0.16 and 0.48) after 144 h of incubation. During stationery phase, the nitrate concentrations continued to drop, although with significantly reduced velocity, the majority of the microbial consortia tested (except AF, PBb, PBs, and S1) removing 100% of the nitrate present in the culture media at the end of the 5 days experimental time (Table 1).

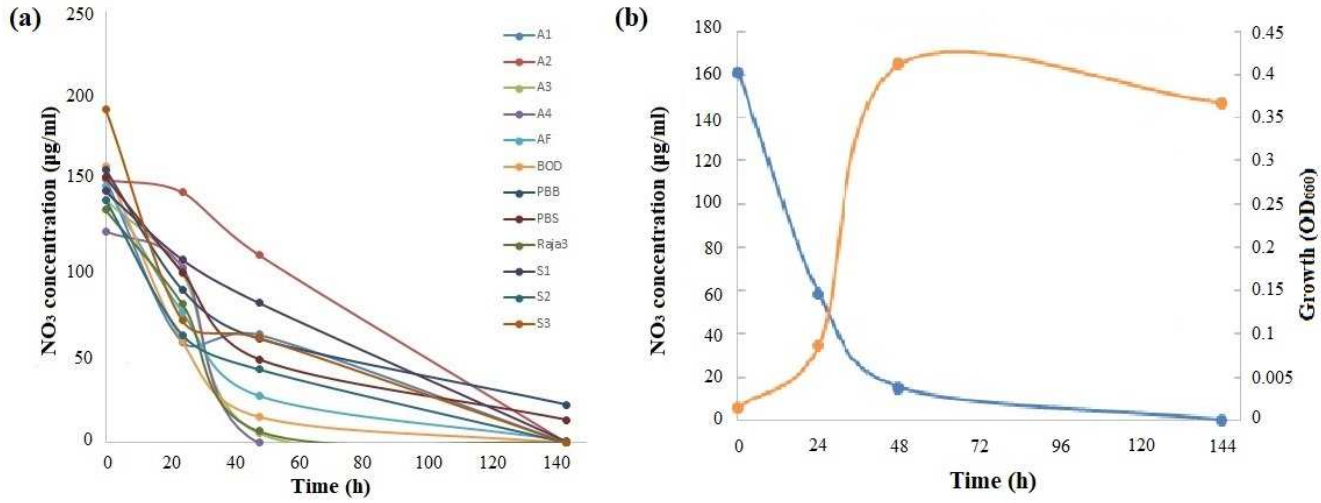

Figure 1. (a) Nitrate removal kinetics and (b) growth curve coupled with the denitrification activity of the BOD microbial consortia

Although all tested microbial consortia exhibited high denitrification abilities, some of them were more efficient than others. The one that separates from the rest as being most efficient throughout all bacterial growth phases was the BOD consortia, being closely followed by A3, A4, Raja3, and S3 consortia. Under aerobic conditions, BOD consortia demonstrated noticeably denitrification performance. During the first 24 h of incubation, the BOD population grew slowly but the NO_3

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removal rate reached the maximum value of 4.24 mg NO_3/L/h. As shown in Figure 1b, the NO_3 decreased from 160.43 to 58.72 mg/L (63.42%) in the first 24 h, but when the BOD consortia entered in its logarithmic growing phase the denitrification rate became weaker (probably due to a low C/N ratio) reducing to a rate of 3.03 NO_3/L/h and levelled off to 1.11 NO_3/L/h when the bacterial growth reached its peak and throughout stationery phase. All NO_3 was completely consumed (160.43 mg/L, 100%) after 5 days of incubation. No NH_4 or NO_2 accumulation was detected in the supernatant during the entire growing phase (data not shown), all NO_3 from the basic medium being converted to biomass nitrogen and gaseous nitrogen. Comparable results were reported for pure cultures of *Marinobacter* sp. F6 (Zheng et al., 2012), *P. stutzeri* YZN-001 (Zhang et al., 2011), and *Pseudomonas* sp. ASM-2-3 (Kariminiaae-Hamedaani et al., 2004) cultivated in similar conditions.

Table 1. Nitrate removal efficiency of the enriched denitrifying microbial consortia isolated from different sources

	A1	**A2**	**A3**	**A4**	**AF**	**BOD**	**PBb**	**PBs**	**Raja3**	**S1**	**S2**	**S3**
Removal rate* (mg/L/h)	1.89	0.91	2.81	2.55	2.54	3.03	2.04	2.20	2.67	1.35	2.05	2.77
Removal efficiency (%)**	100	100	100	100	99.22	100	86.03	91.37	100	99.6	100	100

*The removal rates were calculated after 48h of incubation

**Removal efficiency was calculated at the end of the 5 days experiment

Effect of C/N ratio on nitrate removal

Since carbon is essential for cell growth and denitrification processes, using the optimal amount of carbon versus nitrogen is a decisive factor in the denitrification process (Sobieszuk and Szewczyk, 2006; Krishna Mohan et al., 2016; Chen et al., 2017).

In Table 2 are presented the NO_3 removal rates obtained, after one month of enrichement,for a C/N ratio of 250/1. In version a, 50 mL of activated sludge is suspended in 500 ml reaction volume and in version b, 100 ml of activated sludge is suspended in a 500 ml reaction volume.

Table 2. NO_3 removal rates for a C/N ratio of 250/1

Version	**Ratio C/N**	**mg NO_3removed 50 mL activated sludge/24 h**	**mg NO_3 removed 100 mL activated sludge/24 h**
a	**250-1**	37.23	-
b	**250-1**	-	33.04
a	**250-1**	36.02	-
b	**250-1**	-	31.89
a	**250-1**	35.95	-
b	**250-1**	-	36.13

One can see that smaller volumes of the reaction vessel are more efficient than larger volumes, in our configuration.

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Table 3. NO_3 removal rates for a C/N ratio of 10/1 and 5/1

Version	Ratio C/N	mg NO_3 removed 50mL activated sludge/24 h
a	**10-1**	63.63
b	**5-1**	63.50

In Table 3 are presented the NO_3 removal rates obtained, after one month of enrichement for a C/N ratio of 10/1 (version a) or 5/1 (version b) and a constant volume of activated sludge in both versions (50 mL activated sludge in 500 mL volume of reaction).
The results show that the 10/1 and 5/1 C/N ratios allow the selection, after one month of enrichement, of microbial populations with practically the same denitrification activity. These activities are higher than those obtained when the ratio C/N was much higher (250/1).

4. CONCLUSIONS

In this study, twelve aerobic denitrifying consortia of microorganisms were isolated using enrichment and screening processes. All of them were able to reduce the NO_3 concentration, some of them being more efficient than others. Even so, the efficiency of nitrate removal was of minimum 86% and up to 100% in a 5 days retention time, one of the microbial populations tested (i.e., A4) having a 100% efficiency after 48h.
When it comes to the influence of different C/N ratios on nitrate removal by the enriched endogenous microbiota of activated sludge, lower C/N ratios (10/1 or 5/1) gave better results with respect to denitrification rates, as compared to higher C/N ratio (250/1).
The conducted experiments gave us a clear idea of the effectiveness of each microbial consortia in depleting nitrates from polluted wastewaters. The enriched populations together with expected purified strains will be further studied and used to increase the cleaning/filtration capacities in RAS farms and municipal waste water plants, as well.

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