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ENRICHEMENT OF ACTIVATED SLUDGE IN AMMONIA OXIDIZING MICROORGANSIMS, FOR A MORE EFFICIENT NITRIFICATION IN RECIRCULATING AQUACULTURE SYSTEMS

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Abstract
In the last decades there are reports concerning attempts to try to increase the amount and activity of a given physiological group of microorganisms from the activated sludge, in order to increase the overall yield of wastewater purification. In this paper we report our original results concerning the selective cultivation of microbial populations in growing media specific for ammonia oxidizing microorganisms (AOM), in order to increase the oxidation of ammonia from artificial wastewaters, model for those related to recirculating aquaculture systems. Different microbial populations were cultivated in batch systems at 20 ℃, in aerobic conditions, in media with ammonium as sole electron source and carbon dioxide as sole carbon source. The results show that, after appropriate selective cultivation, there is a significant increase in the activity of AOB, in agreement with the increase in cell densities of AOB estimated by MPN technique. The improved populations thus obtained are good candidates for enhancing the removal of ammonia, firstly at laboratory level, from (synthetic) waste waters. However, the stability/resilience of these populations in true life conditions remains to be evaluated.

Keywords: activated sludge, ammonium oxidizing microorganisms, nitrification, recirculating aquaculture system

1. INTRODUCTION

Aerobic ammonia oxidation is carried out by chemolithotrophic prokaryotes (both bacteria and archaea) which are able to use the oxidation of ammonia (NH_3) or ammonium (NH_4^+) as inorganic substance, in the absence of light, as a source of energy for cell biosynthesis and maintenance (Kelly and Wood, 2013). They are collectively called ammonia oxidizing microorganisms (AOM) participating in the process of nitrification. Nitrification is a major process in the nitrogen (N) cycling, including a two-step process, the oxidation of ammonium nitrogen to nitrite (NO_2) catalysed by ammonia-oxidizing microorganisms (AOM) and subsequently nitrite to nitrate(NO_3) by nitrite-oxidizing microorganisms(NOM). Ammonia oxidation, the first and rate-limiting step of nitrification, has been studied widely because of its ecological significance in the global N cycle and environmental implications (Barnes and Bliss, 1983; Bothe et al., 2000; Kowalchuk and Stephen, 2001; Ofiteru et al., 2010; Shen et al., 2012). As stressed by Vannecke (2015), another possibility lies in the conversion of only half of the ammonium to nitrite (partial nitritation) followed by the conversion of ammonium and nitrite to nitrogen gas in a so-called anaerobic ammonium oxidation (anammox) reaction.

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In the last years, in connection with different types of approaches that are trying to improve the microbial activity in anthropic ecosystems (such as wastewater treatment plants), the ecological view starts to be taken into account by engineers stressing on the importance of higher microbial diversity including greater pool of physiological and genetic traits, which provide the corresponding ecosystems, including wastewater plants, with a higher capacity to change and sustain function under varying environmental conditions (Bellucci et al., 2015). In our opinion, this true ecological view on water purification is in its infancy also in research and practical activities in our country.
Another important idea, related to the above one, concernsthe control of different microbial populations in microbiota (e.g. that of the activated sludge) involved in nowadays depolluting activities (Herrero and Stuckey, 2015; Gerardi, 2016; Nzila et al., 2016) includingin recirculating aquaculture systems.
There are reported in the literature many types of protocols to enrich a given microbiota in AOM, many of them taking into account precise parameters such as the affinity for ammonia or for molecular oxygen or kinetic rates of ammonia oxidation or cellular growth (Bitton, 1994; Boon et al., 2003; Miao et al., 2017). In the framework of aquaculture (Rjin and Rivera, 1990; Colt, 2006; Gutierrez -Wing et al., 2006; Crab et al., 2007), including our experiments concerning ABAWARE project (Moga et al., 2018a; Moga et al., 2018b; Moisescu et al., 2018a; Moisescu et al., 2018b; Moga et al., 2019), the aim of this paper is to use a complex starting microbiological material for the enrichment by selective cultivation in batch conditions of AOM to be further used for wastewater treatment from recirculating aquaculture systems or from wastewater treatments plants.

2. MATERIALS AND METHODS

Biological materialwas a mixture of microbial consortia originated from wastewater plants, home aquaria and microbiota from Frasin fish farm.
Enrichment of the biological material was performed by selective cultivation in the following mineral salt growthmedium (AOM): $(NH_4)_2SO_4$, 5 mM; NaCl, 10 mM; KH_2PO_4, 0.4 mM; KCl, 1 mM; $CaCl_2 \cdot 2H_2O$, 1 mM; $MgSO_4 \cdot 7H_2O$, 0.2 mM; trace element solution, 1.00 ml/L; 0.5 mg/ml cresol red solution, 2 ml/L; HEPES, 20 mM; pH 7.2-7.4 (Bollmann et al., 2011).
Synthetic waste water composition was according to Takaya et al.(2003) diluted 4 times.
Measurement of biological oxygen demand, total and carbonaceous (BOD5) was determined with BOD Direct (Hach Lange LZQ087). The respiratory oxygen consumption was measured in the absence (total BOD) or in the presence (carbonaceous BOD) of the nitrification inhibitor 2-chloro-6-(trichloromethyl)-pyridine (Hach Lange).
Time-dependent consumption of ammonium and nitrate were measured spectrophotometrically on a Specord® 210 Plus (Analytik Jena) using the Spectroquant® reagent test kits (MerckMillipore). The nitrite was analyzed by N-(1-naphthalene)-diaminoethane photometry method at 540 nm.
Estimation of cell density of AOM was carried out by MPN technique via serial dilution, in microplate developed with nitrite reagent after 3 weeks of incubation (Rowe et al., 1977).

3. RESULTS AND DISCUSSIONS

In Figure 1 are presented the results concerning the oxygen consumption by the starting mixed microbial populations, where no tentative of enrichment in AOM has been done. The measurements performed with three volumes of culture on AOM medium (Bollmann et al., 2011), either in the presence or in absence (control) of a specific inhibitor of ammonia oxidation. Logically, with increasing working volumes thereis an increase in oxygen consumption. The important results concerns no differences, for the same working volume, between the O_2consumption in the presence

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or in the absence of the nitrification inhibitor (NI) 2-chloro-6-(trichloromethyl)-pyridine, suggesting that functionally there are no AOM present in our cultures or they are present in very low counts, below the detection limit. Although the experiment was performed with three different culture volumes, still we were unable to detect any differences between the control and the treated samples with regards to the O_2 consumption.

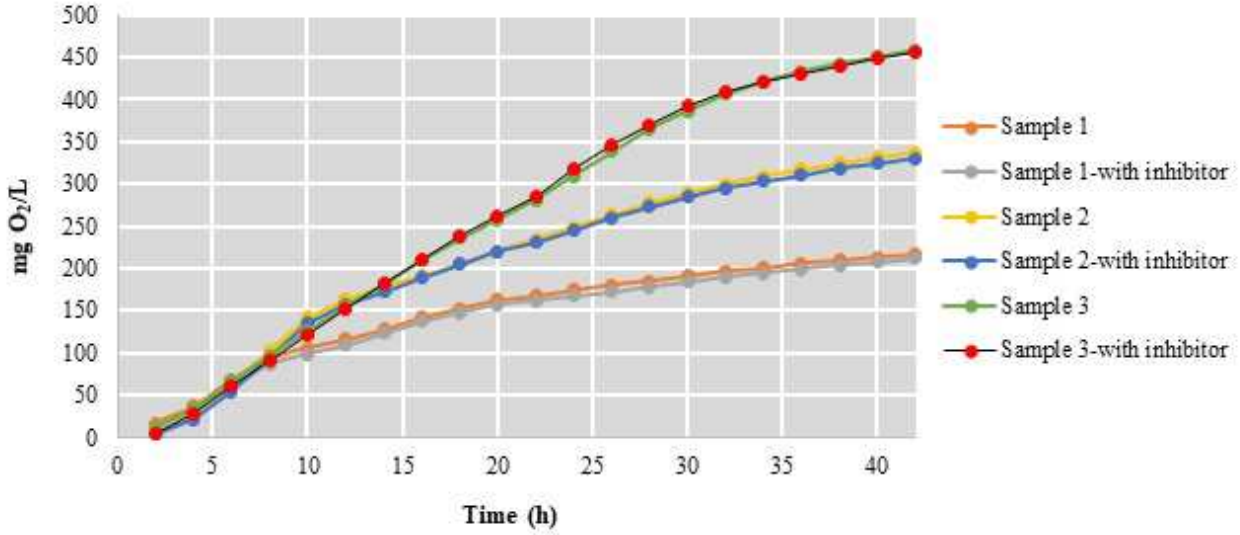

Figure 1. Oxygen consumption by the starting mixed microbial populations, using the same inoculum and 3 different working volumes:sample 1-428mL, sample 2- 244mL, sample3- 157mL

This type of experiment was repeated after the enrichment procedure in AOMwhich consisted in the reinoculation ofthe cultures when the *p*H of the medium became alkaline, as an expression of ammonia oxidation. This process developed for 4 months, with several microbial samples, ended with the selection of one complex consortium which gave reproducible changes in *p*H and decrease in ammonia concentration simultaneous with the increase in nitrite concentration. The results obtained with enriched consortia are presented in Figure 2. One can see that there is a large difference between the O_2consumptionofthe control population (with no NI) and the population enriched in AOM. In the control population, the$O_2$is used for oxidation of organic substrates by both the heterotrophic and the chemolithotrophic AOM. In the presence of the inhibitor, the activity of the AOM population is inhibited and the contribution of the heterotrophic microorganisms is minor. These results clearly argue that in our enriched population the chemolithotrophic AOM are present in large numbers, thus the enrichment protocol was successful.

The efficiency of the enrichment procedure was also tested by the MPN method that estimates the number of viable cells in a given sample. The estimation of AOM cell number in the initial microbiota number showed an increase from 100 cells/mL to 100.000 cells/mL in the enriched microbiota, arguing that there are high differences between the enriched population and the non-enriched (initial) population. Furthermore, as expected, even in the initial non-enriched population there were some AOM present and they were detected by this specific method but their contribution to the O_2 consumption was very small, below the detection limit with BOD device.

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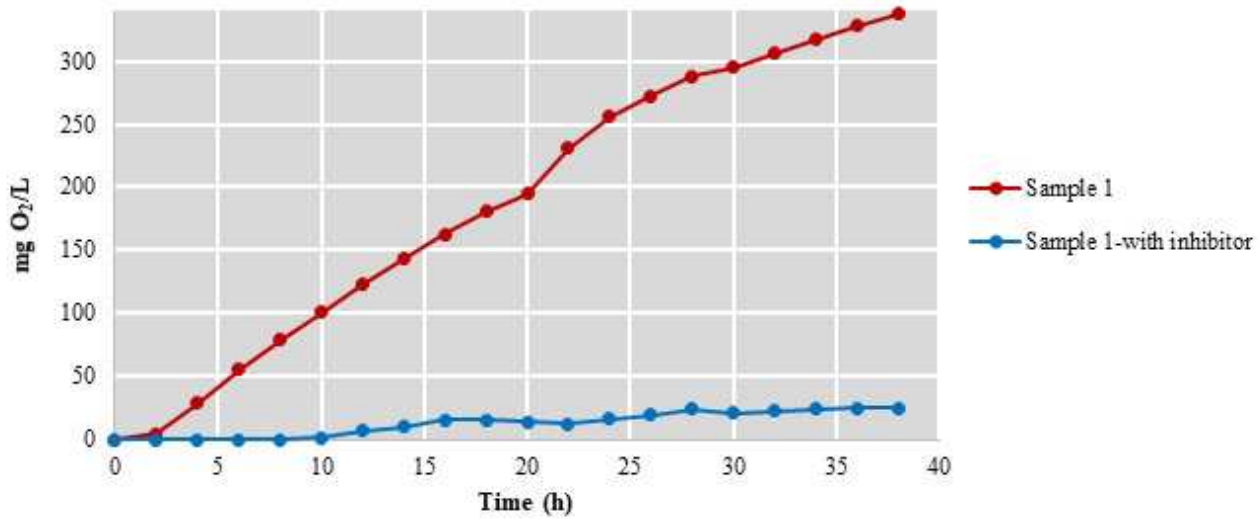

Figure 2. Time evolution of O_2 consumption by the AOM-enriched microbiota, in the presence (blue line) or in the absence of the specific nitrification inhibitor (red line).

In the following experiments, different analyses have been performed in order to test the expected ammonia consumption and the corresponding production of nitrate (via nitrite) by AOM and NOM, respectively. The AOM-enriched microbiota, in the absence or presence of NI were suspended in the same medium containing the same initial concentration of ammonium (9.3 mg/L) and nitrate (92.5mg/L). In Table 1 are presented the results concerning the evolution of nitrate and ammonia concentration in a 5 days experiment by the catalytic action of AOM-enriched microbiota, in the absence of the NI or in its presence.

Table 1. Quantification of ammonia and nitrate in oxygen consumption measurements

	NH_4 initial	**NH_4 final**	**NO_3 initial**	**NO_3 final**
Selected microbiota	9.3 g/L	1.5 g/L	92.5 mg/L	2897.4 mg/L
Selected microbiota with inhibitor	9.3 g/L	1.7 g/L	92.5 mg/L	122.3 mg/L

One can see that, the AOM enriched population, in the absence of the specific inhibitor of ammonia oxidation (Hach Lange LZQ087), induces a decrease in ammonia concentration from 9.3 mg/L to 1.5 mg/L whereas, in the presence of the NI the decrease is only to 1.7 mg/L whereas we observed a high increase in the nitrate concentration.

Furthermore, aiming not only at the enrichment of our cultures in AOM but also thinking at the non- AOM still present in this kind of enrichments, we further divided our enriched population in two subsamples: one maintained in the AOM specific mineral salt growing medium, and the other grown in a more complex medium (AOM medium supplemented with residual synthetic wastewater). The aim was to evaluate how much AO activity remains in the enriched AOM population after 4 months of cultivation in the more complex medium, where other types of microorganisms find energy sources to sustain their cellular growth and multiplication. In this experiment (Figure 3) there are three types of populations: (1)ammonia enriched population grown only in AOM medium and tested in AOM medium;(2)ammonia enriched population adapted for 4 months to grow in AOM medium supplemented with synthetic residual water (SRW), thus

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becoming a complex population; this complex population was tested in AOM medium supplemented with synthetic residual water; and (3) ammonia enriched population grown only in AOM medium but tested for oxygen consumption in AOM medium supplemented with SRW added just before starting the oxygen measurements.

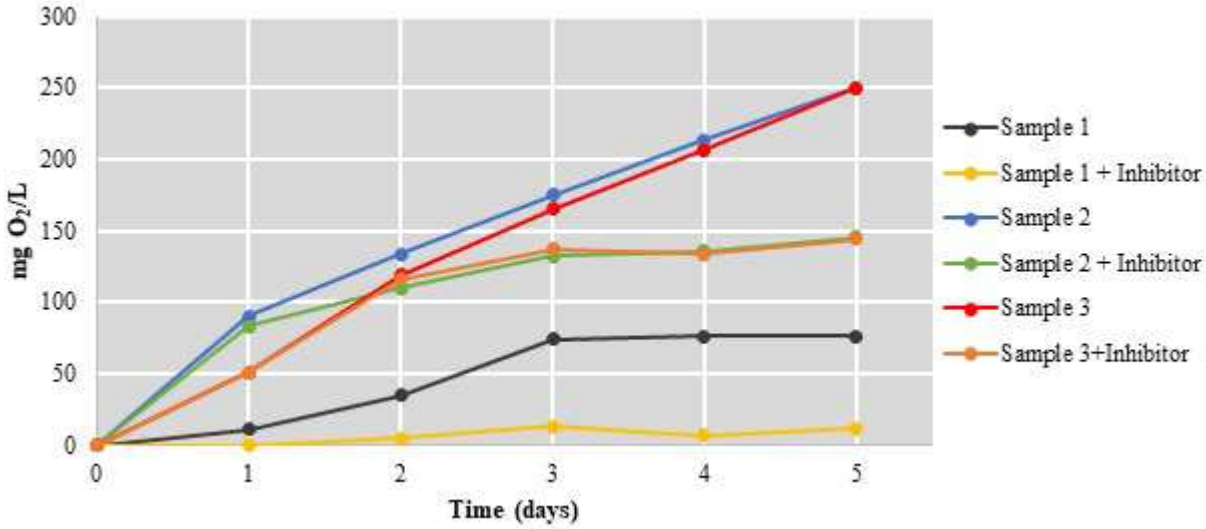

Figure 3. O_2 consumption by ammonia enriched population(Sample 1) and complex populations adapted for 4 months to grow in AOM medium supplemented with synthetic residual water (SRW) (Sample 2), and by enriched AOM population grown in AOM medium supplemented with SRW just before the experiment (Sample 3). All samples were tested either in the absence or in the presence of the NI

The main aim of this experiment was to estimate the capability of non- AOM cells still present in enriched population in AOM to (quickly) metabolically react to the addition of complex organic substance present in either synthetic or true wastewaters, a situation more related to real life conditions found in wastewater treatment plants or recirculating aquaculture systems.
The main conclusion is that oxygen consumption capacity of ammonia enriched population grown only in AOM medium but tested for oxygen consumption (measured as total BOD)in AOM medium supplemented with SRW added just before starting the oxygen measurements (Sample 3) is at the end of the 5 days of measurements, the same with that of ammonia from enriched population adapted for 4 months to grow in AOM medium supplemented with SRW (Sample 2).

4. CONCLUSIONS

a) The use of a simple, batch culture based method, allows the enrichments of complex microbial consortia in ammonia oxidizing microorganisms;
b) These enriched consortia in ammonia oxidizing microorganisms can be maintained in laboratory conditions for further use;
c) Enriched consortia in ammonia oxidizing microorganisms, in the presence of complex organic substances, have the ability to metabolically consume oxygenas that of ammonia enriched population adapted for 4 months to grow in AOM medium supplemented with SRW.

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