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SIMULTANEOUS REMOVAL OF CARBON AND NITROGEN FROM SYNTHETIC MEDIA BY THREE SELECTED BACTERIAL CONSORTIA

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Abstract

Currently, one of the most used and promising purging technique of wastewaters is the biological treatment performed by microorganisms. The ability of three bacterial consortia for simultaneous removal of organic carbon, inorganic nitrogen, and phosphorus was investigated. After 5 days of incubation, the organic carbon (acetate) removal efficiency reached a maximum of 39.24%. Depending on the nitrogen (N) source used, the N removal efficiencies reached 94.69% when using ammonium (NH_4) and 100% when using nitrate (NO_3) as sole N source. No PO_4 removal was detected. Interestingly, the NH_4 reduction seems to be achieved without the accumulation of nitrite (NO_2) or nitrate (NO_3), supporting the idea of simultaneous nitrification and denitrification processes performed by the bacterial consortia. The experimental data showed that all three selected bacterial consortia can efficiently clean the artificial synthetic media with respect to N and C load, being feasible for employment in real wastewater treatment, either domestic or from fish farms.

Keywords: Nitrification, Denitrification, Carbon removal, Bacterial consortia, Wastewater

1 INTRODUCTION

In the last decades there is an increasing interest in controlling the composition of different physiological groups of microorganisms living in the activated sludge, in order to improve the biological purification of spent waters. In fish farms, the main water pollutants are organic matter, ammonia, nitrite, nitrate and phosphorous compounds. Nitrification (the aerobic oxidation of ammonia to nitrite) (Winogradsky, 1890) and denitrification (the microbial process of reducing nitrite and nitrate to gaseous forms of nitrogen) (Skiba, 2008) are the key processes involved in the biological nitrogen removal and an essential component of the wastewater treatment plants (WTPs). It is obvious that nitrification and denitrification are strongly related in nature, being also part of nitrogen cycle. Ammonia can be eliminated by aerobic oxidation (Suwa et al., 1994), anaerobic oxidation (Annamox) (Mulder et al., 1995) or by its assimilation (Neilson and Doudoroff, 1973) by different groups of microorganisms. Two distinct groups of bacteria catalyse the two separate oxidation steps involved in nitrification. The first group is composed of the chemolithoautotrophic ammonia oxidizing microorganisms, which use ammonia as primary source of electrons, thus converting ammonia (NH_3) to nitrite (NO_2^-), while the second group, nitrite oxidizing microorganisms, catalyses the oxidation of nitrite to nitrate (NO_3^-) (Bollmann et al., 2011). Ammonia occurs in waters as unionized ammonia (NH_3) and ionized ammonia (NH_4^+), the first one being highly toxic for aquatic animals even at concentration of 1.5 mg/L (N) whereas nitrite is toxic for salmonids even at 0.2 mg/L (Davidson et al., 2017). Nitrate is less toxic as compared with ammonia and nitrite but its accumulation in different types of fish farms at levels higher than 50 mg/L can cause health problems to fishes (Good et al., 2017). Nitrate removal could be done through different biological processes, either dissimilatory (true denitrifying bacteria use nitrate as final electron acceptor thus reducing it to dinitrogen which is subsequently lost in the atmosphere) (Stouthamer, 1988) or assimilatory (nitrate is used as nitrogen source for anabolic processes) (Pinar et al., 1997). Traditionally, denitrification performed in WTPs is an anaerobic process and therefore relatively expensive to achieve and maintain (Khardenavis et al., 2007). In this paper we describe the ability of three different enriched bacterial consortia, from different origins, to remove ammonia, nitrite and nitrate from synthetic media aiming at selecting consortia able of simultaneous nitrification and aerobic denitrification. These selected consortia could be further used (firstly at laboratory scale level) for bioaugmentation (Raper et al., 2018) which involves the seeding of water-purifying bacteria into aquaculture systems, either as free cells or immobilized on different supports as biofilms.

2 METHODS

2.1 Isolation and enrichment of test microorganisms

Three bacterial consortia were isolated from fish tanks waters of Plutonița fish farm (A3, A4) and from a municipal waste water treatment plant in Constanța county (R3) 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℃, 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.

2.2 Assessment of nitrogen removal performance with different nitrogen sources

To obtain rapid population expansion for the nitrogen removal assay, bacterial consortia were revived by aerobic cultivation for 24 h, at 30℃ in liquid DM medium. Cells were collected by centrifugation at 6500 rpm and 4℃ 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 (OD_{660}) of 1 and inoculated to 1% (v/v) into experimental flasks with a basal heterotrophic medium (g/L): sodium acetate 5; NH_4Cl, 0.23; $MgSO_4 \cdot 7H_2O$, 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. To evaluate the aerobic denitrification capability, $NaNO_2$ (0.29 g/L) and $NaNO_3$ (0.36 g/L) were respectively used instead of ammonium as the sole nitrogen source in the basal medium. The flasks were incubated at 30℃ without shaking. During incubation, samples were withdrawn and the optical density at 660 nm (OD_{660}) was determined to assess the kinetics of microbial growth and the supernatant was used to determine the ammonium, nitrite, nitrate, phosphate, total nitrogen (TN), inorganic carbon (IC), total organic carbon (TOC) concentrations.

2.3 Analytical measurements

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. The IC, TOC, and TN were measured with a Multi N/C 3100 analyzer (Analytik Jena). This analyzer offers the right solution for every application, from examining TOC in drinking and waste water to pharmaceutically used water and purification validation to analyzing TOC, NPOC, POC, TC, TIC and TN_b in surface water. Determination is performed by thermocatalytic decomposition, in the presence of a special catalyst, according to national and international standards. A fully automated analytical process for TC/ TOC/ TNb determination according to EN 1484 and EN 12260 can be applied to save resources and time. The samples are catalytically oxidized at a temperature of 800°C in an oxygen flow. The 16 mm combustion tube, filled with platinum catalyst is used. The formed nitrogen oxides are detected by means of a chemiluminescence detector (alternatively a ChD detector can be utilized), CO_2 detection was done by FR-NDIR. The multi N/C® analyzer was calibrated for total organic carbon (TOC) with a potassium hydrogen phthalate standard solution. A multipoint calibration was used to evaluate the results of the TC/TOC measurement. For total bound nitrogen a calibration was carried out with an ammonium sulphate and potassium nitrate solution according to EN 12260.

2.4 Data analysis

Data in this experiment was analysed by Microsoft Excel software. The nitrification/denitrification rate formula is $(C_0 - C_n)/h$. C_0 is the initial concentration of N source. Cn is the final concentration of N source (NH_4, NO_2 or NO_3) at n hour. h is the time of microbial treatment. The nitrogen 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 N source. The generation time (hours), was calculated by computing the OD_{660} versus time on site http://www.doubling-time.com/compute_more.php.

3 RESULTS AND DISCUSSION

3.1 Nitrate and nitrite removal performances of selected microbial consortia

To investigate the nitrogen removal capacity, three bacterial consortia (A3, A4, R3) were selected from our laboratory collection based on their high denitrification capacity. The selected bacterial consortia were tested regarding their ability to remove three different nitrogen containing compounds such as ammonium (NH_4) and nitrite (NO_2), besides nitrate (NO_3) from a heterotrophic basal culture media with a C/N ratio of 24, under aerobic condition (Figure 1a). All three tested consortia A3, A4, and R3 have shown in a previous study

a high capacity to remove NO_3 from a synthetic culture media (data not showed). In this study, their growth and NO_3 removal characteristics were retested and the results showed that they retained the highest denitrification activity when NO_3 was used as sole N source.

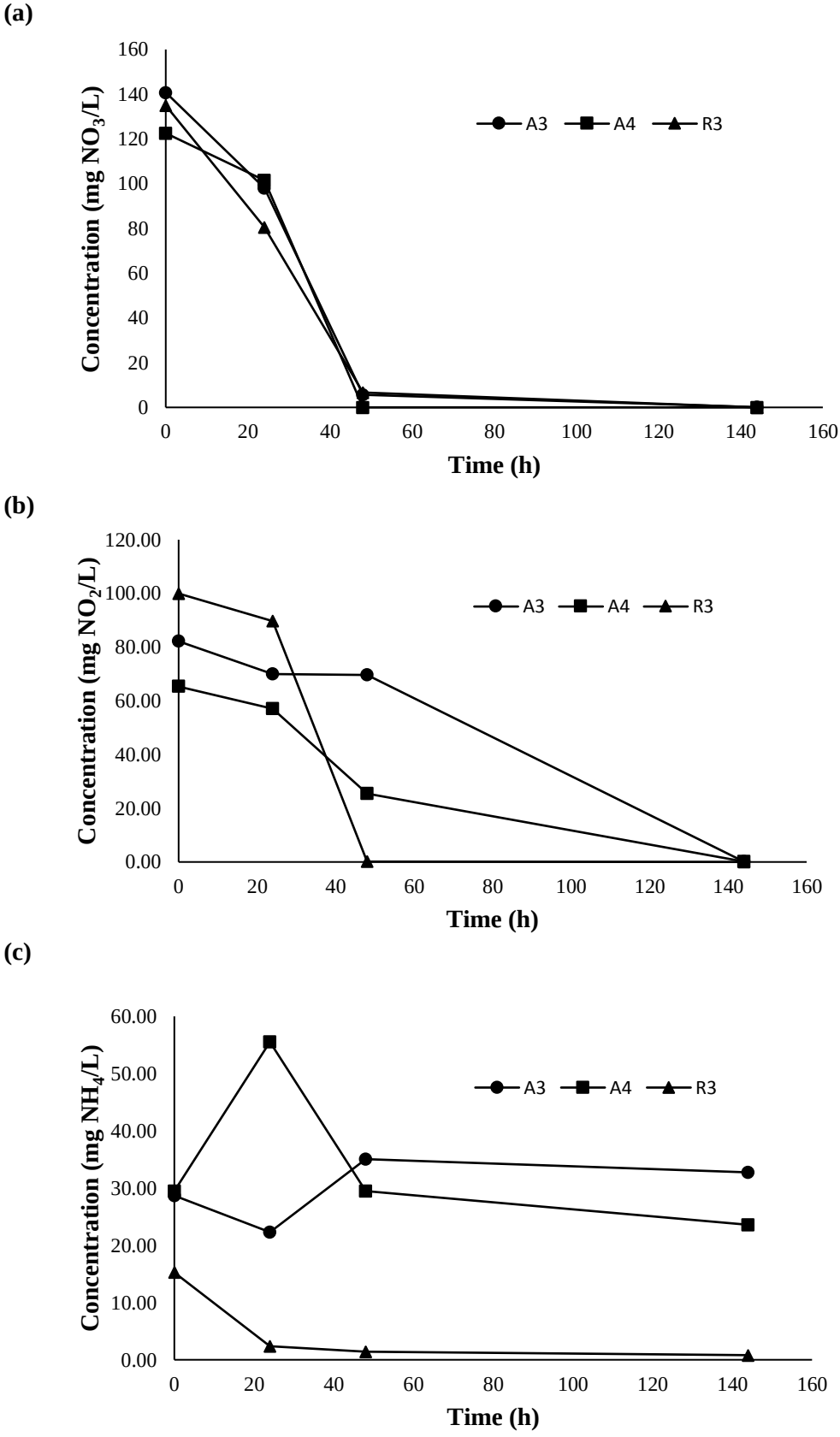

Figure 1. (a) Nitrate (NO_3), (b) nitrite (NO_2), and (c) ammonium (NH_4) removal ability of the selected bacterial consortia

The growth curves of A3, A4, and R3 consortia monitored by OD_{660} were similar in the basal media with NO_3 as N source: a lag phase occurred in the first 24 h, followed by the exponential phase from the 24th to 144 h. Although the growth rate was low in the first 24 h, A3, A4, and R3 consortia were able to remove 30.36%, 17.15%, and 40.35% respectively from the initial NO_3 concentration with a removal rate of 1.78 mg NO_3/L/h, 0.88 NO_3/L/h, and 2.27 NO_3/L/h respectively. In the next 48 h, as the bacterial growth entered the logarithmic growth phase, the removal rates of NO_3 increased to: A3 - 2.81 mg NO_3/L/h, A4 - 2.55 mg NO_3/L/h, and R3 - 2.67 mg NO_3/L/h. When the bacterial growth reached its peak and throughout stationary phase, the NO_3 removal rates continued but with reduced velocities: A3 - 0.98 mg NO_3/L/h, A4 - 0.85 mg NO_3/L/h, and R3 - 0.94 mg NO_3/L/h (Table 1). Similar removal rates of NO_3 were reported in the literature for *Klebsiella pneumoniae* CF-S9 (Padhi et al., 2013) with a removal rate of 2.2 mg/L/h and *Rhodococcus* sp. CPZ24 (Chen et al., 2012) with removal rate of 0.93 mg/L/h. As shown in Figure 1a, the NO_3 was almost completely consumed after only 48 h of incubation (i.e. 95.93%, 100%, and 95.02% for A3, A4, and R3 respectively). At the end of the 6 days of incubation time, the NO_3 was completely (100%) removed by all three bacterial consortia tested (Table 2).

Table 1. Generation time and nitrogen removal rates for different N sources, with acetate as sole C source

N source	Generation time (h)			Removal rates (mg/L/h)		
	A3	A4	R3	A3	A4	R3
NO_3	85.4	45.8	42.59	0.98	0.85	0.94
NO_2	40.58	48.69	50.28	0.57	0.45	0.69
NH_4	32.31	35.43	35.9	0	0.04	0.1

When NO_2 was used as sole N source the growth and removal slopes showed a similar trend, although with slightly reduced optical densities and removal velocities (Figure 1b). The lag phase was longer (up to 48 h) and the measured OD_{660} values were half of those measured when NO_3 was present in the growth media. In the first 24 h, the A3, A4, and R3 consortia removed 14.74%, 12.72%, and 10.28% of the NO_2, with a rate of 0.5 mg NO_2/L/h, 0.35 mg NO_2/L/h, and 0.43 mg NO_2/L/h respectively. At 48 h, the rates reached 0.26 mg NO_2/L/h, 0.83 mg NO_2/L/h, and 2.08 mg NO_2/L/h, the A3 removing 15.31%, A4 - 61.02%, and R3 - 99.91% from the initial NO_2 concentration. As the bacterial consortia approached their stationary growing phase the NO_2 denitrification rates dropped to 0.45 mg NO_2/L/h for A4 and 0.69 mg NO_2/L/h in case of R3, except for A3 were the removal rate reached its maximum value of 0.57 mg NO_2/L/h. At the end of the incubation time (144 h), 99.83%, 99.75%, and 100% of the initial NO_2 concentration was removed by the A3, A4, and R3 bacterial consortia (Table 2).

Table 2. Nitrogen, carbon, and phosphorus removal efficiencies of the selected bacterial consortia

	N removal efficiency (%)			C removal efficiency (%)	P removal efficiency (%)
	NO_3	**NO_2**	**NH_4**	**TOC**	**PO_4**
A3	100	99.83	0	39.24	0
A4	100	99.75	20.04	33.33	0
R3	100	100	94.69	28.24	0

*Removal efficiency was calculated at the end of the 6 days of incubation

3.2 Assessment of ammonium removal capacity

As shown in Figure 1c, the NH_4 removal capacity of A3, A4, and R3 bacterial consortia was not as good as their denitrification capacities. At the beginning of incubation, the concentration of NH_4 in the A3 and A4 consortia had in fact increased, possibly due to the decomposition of the dying cells or possibly by the presence of ammonium producers in the composition of the bacterial consortia. In the case of A3 consortia, the NH_4 concentration had an up-and-down evolution, in the first 24 h decreasing from 29 to 22.3 mg/L with a removal rate of 0.27 mg/L/h, then in the next 24 h increasing to 35 mg/L, to finally drop to 33 mg/L with a rate of 0.02 mg/L/h, a concentration slightly higher than the initial NH_4 concentration present in the growth medium. These results suggest that as some of the NH_4 was removed, other was produced and therefore the

overall NH_4 concentration was maintained to approximately its initial value throughout the entire experimental time. Consequently, the NH_4 removal efficiency was 0% for A3 consortia. A similar situation was observed in the case of A4 consortia where, after an initial increase in the NH_4 concentration from 29.5 to 55.5 mg/L, as the bacterial growth entered in its exponential phase the NH_4 concentration dropped rapidly to its initial value with a removal rate of 1.08 mg/L/h. Afterward, the NH_4 removal continued with a much lower rate of 0.1 mg/L/h until the end of the experiment, such that the NH_4 removal efficiency was of only 20.04% at the end of the experimental time. A much more efficient removal model was recorded in case of R3 consortia, with a maximum removal rate of 0.54 mg/L/h in the first 24 h, which gradually decreased to 0.1 mg/L and settled to 0.04 mg/L/h until the end of incubation. No NH_4 upsurge was noted during the entire incubation for the R3, this consortia being the most efficient (94.69%) in removing the NH_4 from the culture media. Although higher NH_4 removal rates were reported for different bacterial strains (Joo et al., 2005; Zhang et al., 2011; Chen et al., 2012; Li et al., 2015), the advantage of R3 bacterial consortia was that it could simultaneously remove NO_2 and NO_3.

3.3 Carbon removal performances

Carbon is essential for cell growth and denitrification processes, therefore using the necessary amount of carbon versus nitrogen is a decisive factor in N removal processes (Sobieszuk and Szewczyk, 2006). In this study, a batch experiment with acetate as sole organic C source and NO_3 as sole N source was conducted. The C/N ratio used was 24, a relatively high ratio but more than enough so not to hinder the denitrification performances of A3, A4, and R3 consortia tested. Generally, the nitrogen and carbon are simultaneously removed from the culture media. After 6 days of incubation, the total organic carbon (TOC) dropped significantly in all three consortia tested (Table 3), coupled with an increase in the inorganic carbon (IC) represented by the CO_2 produced during cellular respiration. The drop in the TOC concentration was accompanied by a simultaneous removal of total nitrogen (TN) present in the growth media. The efficiency of C removal was of 39.2%, 33.3%, and 28.2% for A3, A4, and R3 consortia. Interestingly is that, although R3 consortia proved to be the most efficient consortia when it comes to removing the N containing compounds (NO_3, NO_2, and NH_4), it showed the lowest efficiency in removing the organic carbon from the culture media. This could be explained if we consider that a percent from the bacterial strains present in the R3 consortia is represented by ammonia oxidizing bacteria, autotrophic microorganisms which do not use organic but inorganic C sources (i.e. CO_2) (Bollmann et al., 2011). This supposition is supported by the results of the present study which showed that the R3 consortia was the most efficient in removing the NH_4 from the culture media.

Table 3. The concentrations of organic and inorganic C present at the beginning and at the end of experimental procedure, with NO_3 as sole N source

	TOC (g/L)		**IC (g/L)**		**TC (g/L)**		**TN (g/L)**	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
A3	0.79	0.48	0	0.14	0.79	0.62	0.05	0
A4	0.78	0.52	0	0.13	0.78	0.65	0.06	0
R3	0.86	0.61	0	0.13	0.86	0.74	0.05	0

4 CONCLUSIONS

In this study, three bacterial consortia (A3, A4, and R3) were tested with respect to their ability to remove nitrogen and organic carbon from a culture media with a composition that can be assimilated to the composition of real wastewaters, either domestic or from fish farms. All three consortia tested were able to simultaneous remove nitrogen and carbon during aerobic denitrification processes, but only R3 proved to be capable of efficiently performing both nitrification and aerobic denitrification. The composition in bacterial species of R3 consortia seems to be the most equilibrate and adequate, making it feasible to be further used for the biological treatment of real wastewaters.

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