SOCIEDAD LATINOAMERICANA DE BIOTECNOLOGÍA AMBIENTAL Y ALGAL

IV SOLABIAA
LATIN AMERICAN
CONGRESS

NOVEMBER 8 TO 13, 2015, FLORIANÓPOLIS, BRAZIL

BIODIVERSITY AND QUANTIFICATION OF FUNCTIONAL BACTERIA IN DEAMMONIFICATION PROCESS

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INTRODUCTION

Since the discovery of anaerobic ammonium oxidation (ANAMMOX) several processes using ANAMMOX activity bacteria have been implemented to improve the autotrophic nitrogen removal in wastewater. Among these processes, the deammonification was recently proposed seeking more effectiveness for the treatment of concentrated effluents with low carbon/nitrogen ratio, as sanitary and domestic sewage or digestate from agricultural wastewater.

Deammonification process combines partial nitritation process with ANAMMOX process, both working together in two (Okabe et al., 2011) or in a single reactor (Zhang, et al., 2012). The deammonification process performed in a single-step could be very economical compared to a two-step process since it requires less operational control and all the nitrogen removal is done in a single reactor (Chang et al., 2013; Zhang et al., 2012). On the other hand, the performance of the single step deammonification process depends on the microbial activity of ammonia oxidizing bacteria (AOB) and ANAMMOX bacteria, both working symbiotically in the same reactor. However, these autotrophic organisms have different preferences for substrate, operating conditions and still, different growing rates, which can cause inhibition or imbalance in the system.

For example, while the AOB demand ammonia and oxygen as source substrate and electrons, the anammox require ammonia and nitrite, and NOB require nitrite and oxygen. When together in the same medium they can disrupt the deammonification process due to competition for oxygen with the AOB and BON, as well as nitrite with ANAMMOX and NOB, and ammonium with AOB and ANAMMOX bacteria. Therefore, the control of bacteria populations is critical to effective deammonification process.

In this way, the molecular biology study can be a valuable tool to better understand and use deammonification technology more efficiently. In this study we aim to characterize and quantify the functional bacterial in a deammonification reactor by FISH analysis (Fluorescent In Situ Hybridisation) and qPCR (polymerase chain reaction in real time).

MATERIAL AND METHODS

Samples were collected in a deammonification reactor (De Prá, 2013) when it was stable activity of the process.

Fluorescent In Situ Hybridisation (FISH)

Hybridization procedures were performed as previously described (Amann et al., 1990) and samples were fixed with para-formaldehyde (4%). Fixed samples were hybridized with different probes specific to each bacteria group, as shown in Table 1, and stained with DAPI (4′,6-diamidino-2-phenylindole). To determine the percentage of organisms, the amount of cells hybridized with specific probe were compared with DAPI stained cells amount. For visualization, an epifluorescence microscope (Olympus BX41, USA) was used.

Bacteria quantification by polymerase chain reaction in real time (qPCR)

Real time PCR (qPCR) analyses were used to quantify the concentration of specific catabolic genes linked to nitrification (amoA) and anammox (HZO) activity, as described by Rotthauwe et al. (1997) and Schmid et al. (2008), respectively. qPCR reaction mixtures containing 2x qPCR-SYBR-Green mix (Ludwig Biotec, Brazil), 500 nM forward and reverse primers (Prodimol Biotecnologia, Brazil), sterile DNase-free water (Ludwig Biotec, Brazil) and 2µL DNA sample, were submitted to qPCR analyses utilizing a Rotor Gene 6000 equipment (Corbett Research, NSW, Australia). Each sample was carried out in duplicate, and on each plate four serial dilutions of standard were run in triplicate for each assay (Angnes et al., 2013). The genome copies (gc) were defined as the mean of the data obtained. Ultrapure water was used as non-template control for each assay.

RESULTS AND DISCUSSION

Figure 1 shows the FISH analysis results for deammonification reactor sample. We can see that the sample was positive for all the specific bacteria evaluated. Among the genera found, Figures 1B and 1E call attention to the strong staining and more defined groups in larger quantities. The first one comes to ammonia oxidizing bacteria (AOB), in which observing Figure 1C is noted that most are composed of the Nitrosomonas genus. The second it is ANAMMOX bacteria, mainly responsible for the elimination of nitrogen during deammonification process. These two bacteria corresponded to most of the total bacteria (Figure 1A) analyzed during the experiment, which indicates a very enriched culture for the occurrence of deammonification process.

Furthermore, as shown in Figure 1D, the reactor was positive - even in small quantities - for the presence of nitrite-oxidizing bacteria (NOB) group, which the Nitrobacter part. This can lead to nitrate formation surplus (resulting from the activity of these bacteria), which can therefore contribute to the reduction of nitrogen removal efficiency in the system. However, although present, the NOB bacteria can be inhibited through the operating parameters applied to the system, such as oxygen limitation dissolved as a strategy to control these populations (De Prá, 2013).

Likewise, the qPCR results corroborate the results of FISH, since quantitatively showed a population of 10^{11} g bacteria^{-1} nitrifying (*amoA*) and 10^{8} cg g^{-1} ANAMMOX bacteria (*hzo*). Also, the qPCR analysis indicated that the biodiversity of the reactor DMX consisted mostly by bacteria oxidizing ammonia and ANAMMOX bacteria as dominant functional.

Liu et al., (2012) found that the AOB bacteria was 10^{8} to 10^{10} bacteria per mg biomass in a deammonification reactor. Still, the increase and decrease of the AOB cell number was directly related to the increase or reduction of N-NH_3 concentration in the system input, which did not happen for the ANAMMOX bacteria, which remained around

10^7 throughout the experiment. However, it may be related to the long duplication of ANAMMOX bacteria (9 to 11 days) and not necessarily the change in ammonia concentration.

If we relate to the growing rate of these functional bacteria, we conclude that AOB populations are commonly found in greater quantity than ANAMMOX populations when subjected to the same reactor during deammonification process. Nevertheless, through the operation control of the process parameters, it is possible to maintain the balance of these populations, making the overall efficiency of nitrogen removal is not affected by the difference between these results.

As previously mentioned a good balance between the BOA and the anammox bacteria is very important to control deammonification process. Changes in external factors can disturb the environment, leading to decreased activity and possible cell death of functional bacteria. Therefore, to characterize qualitatively and quantitatively the biomass responsible for the nitrogen conversion in the reactor are fundamental to future technological application of this process.

CONCLUSIONS

The FISH and qPCR analysis demonstrated that the functional dominant bacteria present in the reactor are AOB (1011 bacteria g-1) and ANAMMOX bacteria (108 bacteria g-1), which makes the reactor culture rather enriched to deammonification process. Moreover, biodiversity of other bacteria found in reactor demonstrates that while not having its favored activity, there is a potential for development of other biological processes in the system.

Table 1. Sequence and probes specific used in FISH analysis for deammonification reactor samples.

Probes	Sequence 5'- 3'	Specificity
EUB mix (EUB338 I + EUB338 II + EUB338 III)[a]	GCT GCC TCC CGT AGG AGT GCA GCC ACC CGT AGG TGT GCT GCC ACC CGT AGG TGT	All Bacteria
Nso190[b]	CGA TCC CCT GCT TTT CTC C	All AOB
NEU[a]	CCC CTC TGC TGC ACT CTA TTC CAT CCC CCT CTG CCG*	Nitrosomonas
NIT3[c]	CCT GTG CTC CAT GCT CCG CCT GTG CTC CAG GCT CCG*	Nitrobacter
AMX368[d]	CCT TTC GGG CAT TGC GAA	All ANAMMOX

[a]POLPRASERT e SAWAITTA-YOTHIN, 2006; [b]MOBARRY et al., 1996; [c]WAGNER et al., 1996; [d]SCHMID et al., 2003; *Probes competitors.

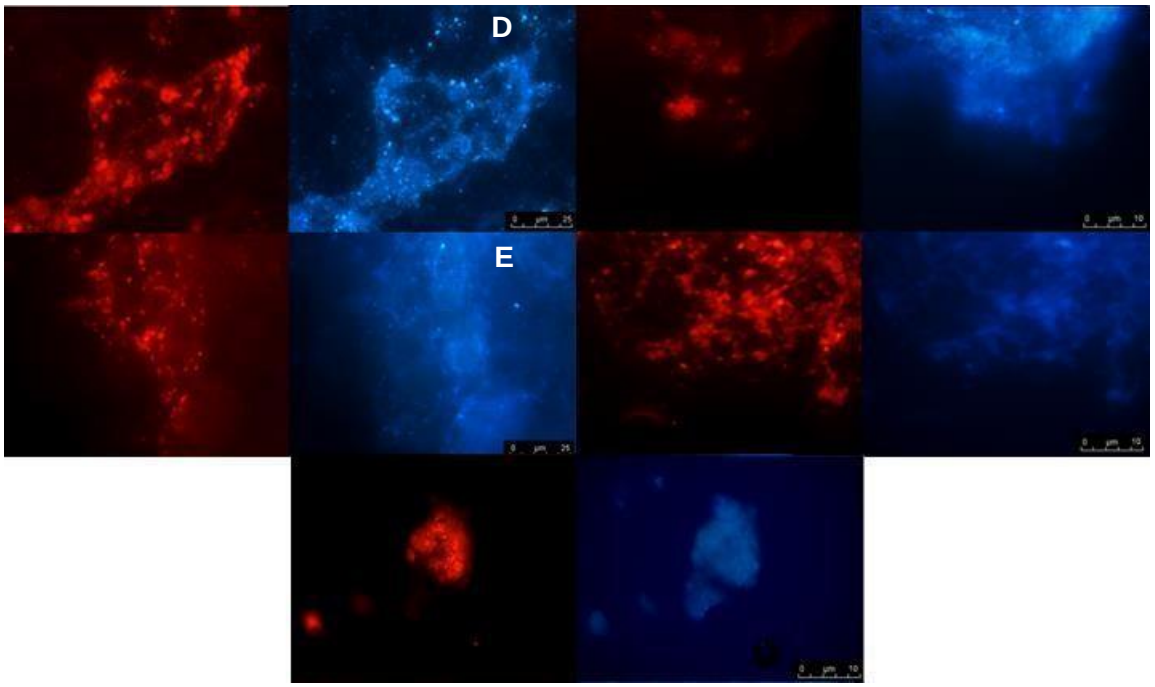

Figure 1. Blue: DAPI staining of the total bacterial community. Blue: DAPI staining of the total bacterial community. Red: staining of specific bacteria, where (A) All bacteria; (B) All AOB bacteria; (C) *Nitrosomonas;* (D) *Nitrobacter*; (E) All Anammox bacteria.

ACKNOWLEDGEMENTS

Authors thanks financial support from CAPES and CNPq.

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