The Intertrophic Relationship between Algae and Bacteria from the Activated Microalgae Granules

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Activated microalgae granules, as a technology at its initial steps, require sustained efforts for a better understanding of the intertrophic relationship between algae and bacteria. The activated algae granules samples from extensive lab-scale sequencing batch reactor experiments were selected for a total DNA extraction and analysis. A relative quantitative analysis of bacterial genes involved in nitrification, denitrification, phosphorus accumulation as well as genes belonging to microalgae was performed by real-time PCR / qPCR. Microscopic investigations of activated microalgae granule showed a gradual decrease of microalgae concentration, together with an increase in bacterial populations inside the granule.

Keywords: microalgae, bacteria, activated sludge

Wastewater treatment importance is given by the need of increasing the quality of effluents prior their discharge into environment, in order to reduce the pollution impact on the receiving bodies [1]. In the past years, pollutants loads in wastewater have increased due to population increase and industrialization. Conventional wastewater treatment plants rely on physico-chemical and biological processes (most often activated sludge) [2, 3] for organics and nutrients removal. Due to the need for nutrients removal, the activated sludge systems operation and design has increased costs (for pumping and external aeration, aeration alone represent approximatively 50 % of all power costs of a wastewater treatment plant), resulting in the need for identifying new cost-efficient solutions that can lead to effluents complying to the legislative requirements [4,5]. Moreover, at the present, the current by-products of activated sludge systems are mostly considered and treated as wastes.

Wastewater treatment costs must be signiûcantly reduced while maintaining an increased treatment efficiency and a reduced environmental impact [6]. Thus, a new algae-based technology emerged as a better option for wastewater treatment with a low cost and high efficiency, generating useful products instead of excess sludge. The main challenge for the use of microalgae in wastewater treatment is algae harvesting [7] due to their settling characteristics and operational conditions. The limitations identified so far for using symbiotic aggregates of microalgae and activated sludge have included biomass composition control difficulties, (that can be obtained by the selection of the desired species), identification of an algae and bacteria biomass optimal ratio, micro-pollutants removal efficiencies and the potential need for external CO_2 source. Reports on algae-bacteria interactions [8- 11] and studies on the influence of basic operational parameters: hydraulic retention time, organic loading rate [12] or light intensity [13] showed the potential of using activated algae granules in wastewater treatment.

The Workshop on Microalgal Based Wastewater Treatment Facilities at the 13th IWA Leading Edge Technology (LET) Conference on Water and Wastewater Technologies provided experts the opportunity to share their findings and set research priorities to resolve remaining challenges around microalgae's applicability. This year, from the 27th to the 31st of May 2018, LET documented recent developments and applications on Microalgal Based Wastewater Treatment Facilities (www.iwa-let.org).

Another important parameter of the wastewater treatment is nitrogen compounds removal by nitrification-denitrification. Nitrification is the biological oxidation of ammonium to nitrate. Two groups of organisms contribute to ammonia oxidation to nitrite :ammonium-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). The nitrification capacity of ammonia-oxidative bacteria relies on an enzyme system consisting of ammonium monooxygenase and hydroxylamine oxidoreductase. The enzymes role is to catalyze ammonium oxidation to nitrite via hydroxylamine as a intermediary compound. Further oxidation is catalysed by nitrite oxidoreductase (representing the most appropriate functional marker specific for nitrite-oxidant bacteria).

This paper analysed the intertrophic relationships between microalgae and bacteria in terms of metabolic dependencies as an essential data for further development of a new energy efficient wastewater treatment technology.

Experimental part

DNA extraction and amplification

Briefly, the microalgae-bacterial biological samples were collected and the extraction of genetic material (total DNA) was performed using a DNA extraction kit (MoBio PowerSoil DNA isolation kit). The obtained genetic material was purified and quantified at 260 nm wavelength using L-VIS plate (CLARIOSTAR microplate reader). Quantitative analysis of presence and expresion of certain genes belonging to bacterial populations involved in nitrification, denitrification, phosphorus accumulation as well as genes belonging to microalgae was performed by real-time PCR / qPCR (table 1).

Microscopy

The granules structure and morphology were observed by advanced techniques of confocal laser scanning microscopywith automatic XYZ scanning.

	Target	Nucleotide sequence (5'-3')
	Primer	
1.	16s rRNA *Nitrospira*	
	*nsr*1113f	CCTGCTTTCAGTTGCTACCG
	*nsr*1264r	GTTTGCAGCGCTTTGTACGC
2.	*Nitrosomonas*	
	amo 598f	GAATATGTTCGCCTGATTG
	amo 718r	CAAAGTACCACCATACGCAG
3.	Ammoniu monooxigenase (AMO)	
	Amo A 1F	GGGGTTTCTACTGGTGGT
	Amo A2R	CCCCTCKGSAAAGCCTCCTCC
4.	16sRNA AOB (amonium oxydation bacteria)	
	CTO189FA/B[a]	GGAGRAAAGCAGGGGATCG
	CTO189FC[a]	GGAGGAAAGTAGGGGATCG
	CTO654R	CTAGCYTTGTAGTTTCAAACGC
5.	16s rRNA *Nitrobacter*	
	*fgps*872	TTTTTTGAGATTTGCTAG
	*fgps*1269	CTAAAACTCAAAGGAATTGA
6.	Nitrite oxydoreductase gene (nxrB)	
	*nxr*B 1F	ACGTGGAGACCAAGCCGGG
	*nxr*B 1R	CCGTGCTGTTGAYCTCGTTGA
7.	Nitrite reductase (nirK)	
	nir K1F	GGMATGGTKCCSTGGCA
	nir K5R	GCCTCGATCAGRTTRTGGTT
8.	Nitrite reductase (nirS)	
	nirs CD3AF	GTSAACGTSAAGGARACSGG
	nirs R3	GASTTCGGRTGSGTCTTGA
9.	NO reductase	
	cnorb-2F	GACAAGNNNTACTGGTGGT
	cnorb-6R	GAANCCCCANACNCCNGC
10.	N₂O reductaza	
	*nosz*F	CGYTGTTCMTCGACAGCCAG
	nosz 1622 R	CGSACCTTSTTGCCSTYGCG
11.	Phosphorus accumulatig bacteria (PAO)	
	pao462f	GTTAATACCCTGWGTAGATGACGG
	pao651r	CCCTCTGCCAAACTCCAG
	pao846r	GTTAGCTACGGCACTAAAAGG
12.	Universal- bacteria	
	341 F	CCTACGGGAGGCAGCAG
	515R	AATCCGCGGCTGGCA
13	bisphosphate carboxylase	
	rbcL-M379 F	GGTTTCAAAGCTYTWCGTGC
	rbcLFP R	GTAAATACCACGGCTACGRTCTT
14.	universal chlorophyte primers	
	UCP4 F	ACGATCTAAAAAMGCATACAT
	UCP4 R	AATTGTWTCDTTDGCACCDGAAG
15.	Universal: (bacteria and cyanobacteria)[b]	
	CYA106F	CGGACGGGTGAGTAACGCGTGA
	CYA359F	GGGGAATYTTCCGCAATGGG
	CYA781Ra	GACTACTGGGGTATCTAATCCCATT
	CYA781Rb	GACTACTGGGGTATCTAATCCCTTT

[a] Mixture of CTO 189fA/B and CTO 189fC in weight ratio of 2:1
[b] Forward primers CYA 106F and CYA 359F were used in alternative reactions with reverse primers
CYA 781Ra and CYA 781 Rb in echimolar ratio

Samples	A260nm (ng/microL)	A260/280
X1	23.83	1.8
X2	12.16	1.6
X3	20.59	1.6
X4	19.43	1.75
X5	22.29	1.8
X6	21.92	1.8
X7	21.28	1.7

Results and discussions

The microalgae-bacteria granules were collected at different time intervals from a lab-scale sequencing batch reactor where the treatment process was carried out in the absence of external aeration, the oxygen requiered by the aerobic bacteria had being supplied exclusively by the photosynthesis process carried out by photoautotrophic microalgae in the light phase.

The DNA extraction and quantification of the seven microalgae-bacteria granules samples showed no significant difference in extraction yield (table 2).

To assess the microbial diversity and evolution of microbial populations (microalgae-bacteria) over time and under different operational conditions, specific and quantitative analysis by real time PCR were performed. 100ng of DNA was amplified in the presence of the above-mentioned primer sets following the protocol: a) denaturation of the initial DNA template (10 min, 95 °C); b)

40 amplification cycles of denaturation at 95 °C time 15 seconds, followed by 60 seconds specific binding of the primer template AND template and an amplification at 68 ° C). Seven samples were chosen specifically based on the nitrification / denitrification (figs. 1, 2 and 3) and phosphorus (fig. 4) removal performances and following significant variations of the operational parameters to evaluate the effect on the microbial populations in the activated microalgae granules.

Denitrification is the biological process by which nitrate is reduced gradually to nitric oxide (NO), nitrogen oxide and, finally, gaseous nitrogen using a complex enzymatic system coded by specific genes (NirK or NirS, NorB and NosZ). Reduction of nitrite, catalyzed by nitrite reductase (Nir) is the key step in the denitrification process. Two different structurally similar but functionally equivalent nitrite reductases, which are translated from the nirS and nirK genes, are considered to be the predominant enzymes for catalysing nitrite reduction. Overall, the results showed no major difference between NH_4^+-NO_2^- nitrifying

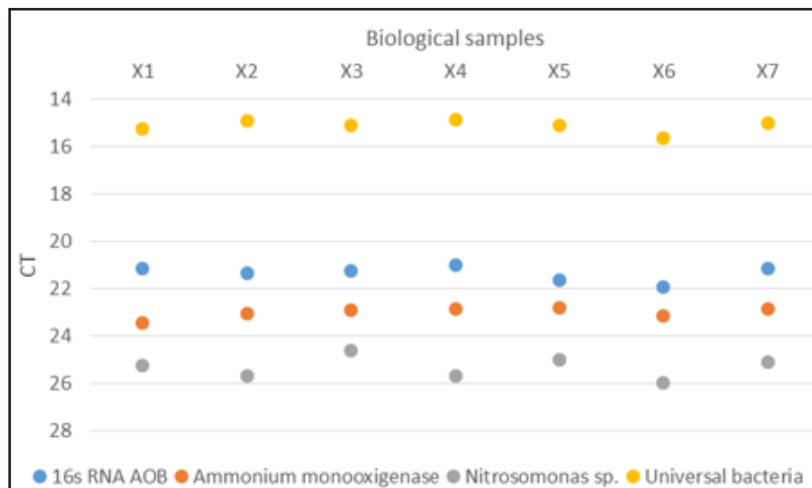Fig. 1. Diversity and dynamics of nitrifying microorganisms (NH4-NO2)
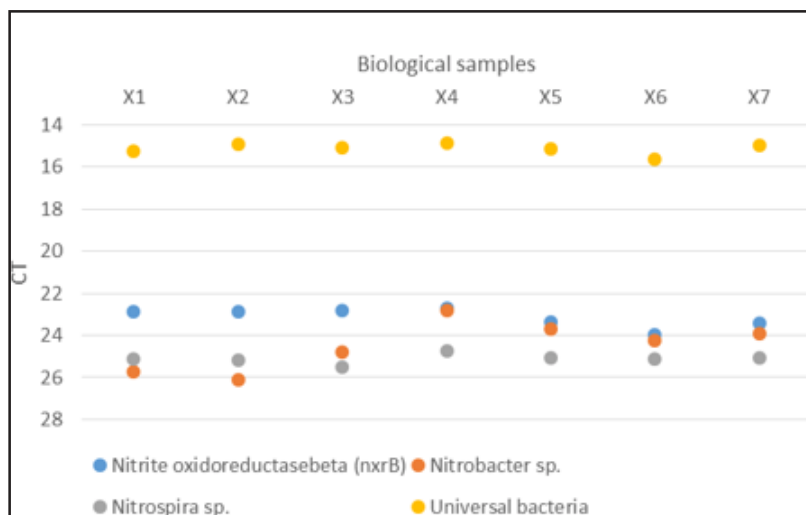

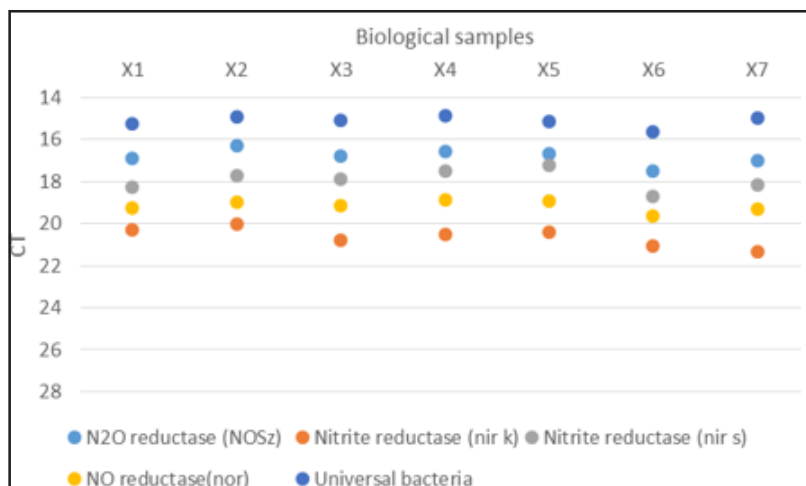Fig. 2. Diversity and dynamics of nitrifying microorganisms (NO_2-NO_3)

Fig. 3. Dynamics of genes involved in the synthesis of nitrogen oxides reductases corresponding to denitrifying heterotrophic microorganisms
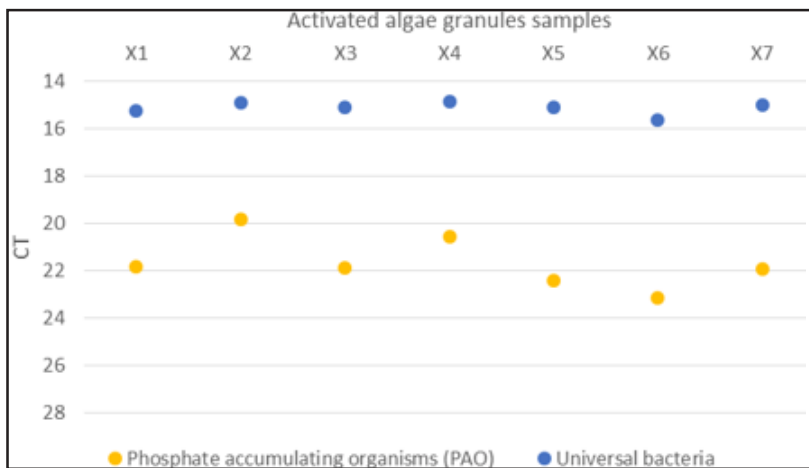
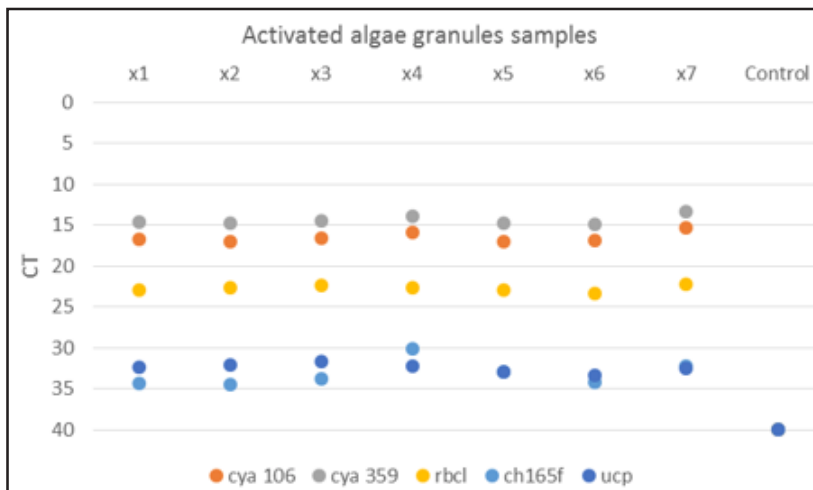Fig. 4. Dynamics of microorganisms able to accumulate phosphorus (PAO)

Fig. 5. Diversity and dynamics of photosynthetic microorganisms and the share of total microorganisms
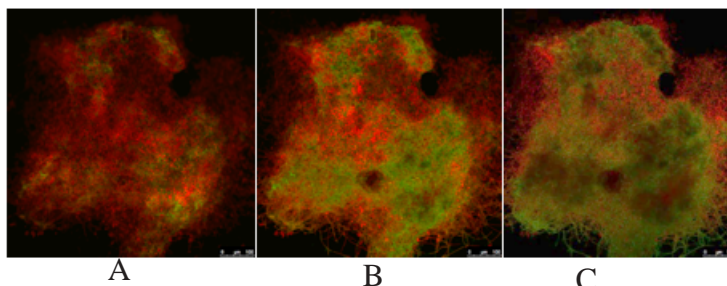

Fig. 6. Microalgae (red) - bacteria (green) granules: A) -0.3 µm from the surface of the granules; B) -6.9 µm from the surface of the granules; C) -17.4 µm from the surface of the granules

microorganisms (fig. 1), a certain balance between species/genes being observed for all samples. Within the granules, among the NO_2^--NO_3^- nitrifying microorganisms Nitrobacter sp. encountered favorable conditions for development and enrichment (fig. 2) in the biological samples collected after a longer period of maturation (biological samples X4 to X7). The abundances of gene copy numbers N_2O and nitrite reductase showed reduced variations over time (fig. 3) and correlates with the universal bacteria showing that a large portion of the bacteria in the granules are heterotrophic denitrifies.

The results have shown an important share of phosphorus accumulation microorganisms (fig. 4), PAOs representing about 25% of the total microorganisms (Universal bacteria) in the activated microalgae granules. Also, the diversity and dynamics of photosynthetic microorganisms from total microorganisms (fig. 5) results showed that chlorophyll (ch165 / ucp) microorganisms have the smallest share in activated algae granules, followed by photosynthesis (rbcl) compared to total cya present in microalgae-bacteria granules.

Microscopic investigations of activated microalgae granule (fig. 6) showed a gradual decrease of microalgae concentration, together with an increase in bacterial populations inside the granule (focusing minus 0.3µm from

the granule surface) towards the interior of the granule (minus 17.4 µm from the granule surface) .

The results correlated with the metabolic processes through which the microalgae use the sun's energy and synthesize the metabodies that are used by the bacteria embedded inside the granule. Also, the emission observed in figure 6 in the range of 500-540 nm is exclusively due to the dye and not to the chlorophyll.

Conclusions

The induced symbiosis between microalgae and bacteria led to an activated microalgae granular structure where bacteria was surrounded by microalgae. This structural configuration fits the hypothesis that microalgae use the CO_2 from the bacterial biodegradation of organic matter while microalgae, through photosinthesis produces the O_2 which is used at its turn by the bacteria in the biodegradation processes.

The activated microalgae granules have a dynamic structure which adapts to the influent composition and operational conditions. Overall, the use of the microalgae-bacteria symbiotic relationship could be an option to replace the conventional activated sludge used in the wastewater treatment.

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