BIOREMOVAL OF NUTRIENTS, ORGANIC CARBON AND PHARMACEUTICALS USING AEROBIC GRANULATION

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ABSTRACT

Biological treatment has been identified as a critical step in the removal of pharmaceuticals from wastewater during the treatment process. Aerobic granulation systems (AGSs) represent an upcoming biological treatment technology for consolidated removal of carbon, nitrogen and phosphorous from wastewater with a smaller footprint. However, the capacity of AGSs to remove organic micropollutants has so far been poorly characterized.

The overarching goal of this study is to determine the effects of the primary carbon substrate on the biotransformation of six ubiquitous micropollutants in AGSs. Acetate, 2-propanol and glycerol were used as the primary carbon source for three AGSs. The different carbon sources did not affect the removal of conventional contaminants (C, N and P) but significantly influenced the removal of individual organic micropollutants and taxonomic richness within the granule. Overall, simultaneous C, N and P were removed at 75 ± 10% and the bulk of micropollutants were (ug l^{-1} VSS^{-1}): 21 ± 35, 36 ± 20 and 60 ± 43 for with acetate, propanol and glycerol grown granules, respectively. Richness (OTUs): 4602, 3017 and 2595 for acetate, propanol and glycerol grown granules, respectively

This study demonstrates that AGSs developed for wastewater treatment can effectively remove micropollutants without compromising the C, N and P removal performance.

KEYWORDS

Simultaneous nutrient and organic matter removals, pharmaceutical compounds, aerobic granules.

1 INTRODUCTION

Wastewater treatment plants (WWTPs) are now widely acknowledged as a major point source for the release of organic micropollutants such as pharmaceuticals to surface waters (Loganathan *et al*., 2009; Spongberg and Witter, 2008). Current municipal WWTPs are not designed to remove micropollutants. Moreover, their low concentration and diversity not only complicate the associated detection and analysis procedures but also create challenges for water and wastewater treatment processes (Luo *et al*., 2014). Biodegradation is considered to be one of the most promising clean-up technologies due to its low cost and its potential for complete degradation of pollutants. Biodegradation of micropollutants is mainly attributed to co-metabolic activities of both heterotrophic and autotrophic microorganisms. Unlike conventional organic pollutants (e.g. COD), many of the organic micropollutants are toxic or resistant to microorganisms and they are present in trace concentration (ng l^{-1}-ug l^{-1}).

Aerobic granular sludge (AGS) is regarded as one of the most promising and versatile technologies in the area of biological wastewater treatment (de Kreuk and van Loosdrecht, 2006). AGS technology for combined carbon, nitrogen and phosphorous removal is based on a repeated fed batch process and relies on microorganisms selected to growing granules rather than flocs. As a result of the high settling rate of the sludge granules, separate settling tanks are not required and an 80% reduction in area use is possible (Pronk *et al*., 2015). Aerobic granules present in their structure zones where different environmental conditions exist. Due to this structure, aerobic granular sludge can simultaneously remove phosphorus, nitrogen and COD (chemical oxygen

demand) from the liquid (de Kreuk *et al.*, 2005). Granular sludge provides many ecological niches due to substrate gradients. Also granules can harbor slow growing organisms due to higher biomass retention time and an applied feast feminine regime (de Kreuk and van Loosdrecht, 2004). Falas *et al.* (2012) show that several pharmaceutical products have significantly higher removal rates with the biofilm compared to the flocculent sludge. AG share many features of biofilm systems. However, in spite of this, little is still known about the granular biomass capacity to remove pharmaceuticals in biofilm systems.

Thus, in this study, we investigated whether the aerobic granular sludge could have higher pharmaceutical removals if the primary carbon source decreases in complexity (hard to easy to degrade). The primary growth substrates in biological treatment systems can suppress micropollutant transformation rates and act as microbial selectors (Falas *et al.*, 2016). Besides that, number of taxa (ie. taxonomic richness) is one ecological factor that might be important for the performance of the WWTP microbial community, so that communities that contain more taxa are more likely to have more functional traits (i.e. biotranformation of pharmaceutical compounds). The selected groups of micropollutants are representative of the main groups of pharmaceuticals and they have different removal rates in conventional WWTPs. Table 1 shows the different pharmaceuticals selected and the corresponding group were they belong to. This table also summarizes the occurrence data of pharmaceuticals in WWTPs influent and their removal percentage across the world as well as their impact on the water cycle (high priority list). In general, IB reported removal rates are among the highest ones of all pharmaceuticals. Also, as can be noted from the table, the reported concentrations and removals reveal significant variations. Aerobic granular formation and shock with IB, NPX, SMX, TMP, TYL and CBZ are explained in section 2 and 3 respectively.

Table1. Pharmaceutical concentrations and removals in conventional WWTPs, their impact on water and the bacteria related to their biodegradation.

Categories	Selected compounds	High priority risk in water [a]	Influent ($ug\ l^{-1}$) [b]	Removal (%) [b]	Bacteria related to degradation	
					Metabolism	Co-metabolism
Anti-inflamatory	Ibuprofen (IB)	Yes	<0.004-603	72-100	Sphingomonas, Ibu-2; patulibacter	Heterotrophs
	Naproxen (NX)	Yes	<0.002-52.9	43.3-98.6	-	Stenotrophomonas maltophilia, planococcus
Antibiotic	Sulfamethoxazole (SMX)	Yes	<0.003-0.98	4-88.9	-	Rhodococcus, microbacterium
	Trimethoprim (TMP)	No information	0.06-6.8	<0-81.6	Heterothrophs in nitrified activated sludge	
	Tylosin (TYL)	No information	-	-	-	Citrobacter Amalonaticus
Anticonvulsant	Carbamazepine (CBZ)	Yes	<0.04-3.78	<0-62.3	-	streptomycetes

[a]de Voogt *et al*. (2009); [b]Luo Y. *et al*. (2014)

2 AEROBIC GRANULAR FORMATION.

2.1 METHODS

2.1.1 EXPERIMENTAL SET UP AND REACTOR OPERATIONS

Experiments were performed in three glass column-type aerobic granular sludge sequencing batch reactors named SBR_A; SBR_P; SBR_G using acetate, 2-propanol and glycerol as a primary carbon source respectively. The reactors were operated in a 3 h time cycle with a 8 min feeding period, 60 min anaerobic, 112 min aeration, 3 min settling time and 5 min effluent withdrawal. The volume exchange ratio was 50%, resulting in a hydraulic retention time of 6 h and a working volume of 200 ml. Aeration and nitrogen were supplied through an air diffuser placed in the bottom of the reactors (airflow rate of 4 l min^{-1}). Each reactor was inoculated from granular sludge from a 2 l sequencing batch granular reactor named SBR_1 inoculated with fresh activated sludge from Rotorua WWTP and acetate and methanol as carbon sources. The reactors were fed with synthetic wastewater daily made, consisting in COD of 900 mg l^{-1} and ammonium concentration of 60 mg N l^{-1} in the feeding media.

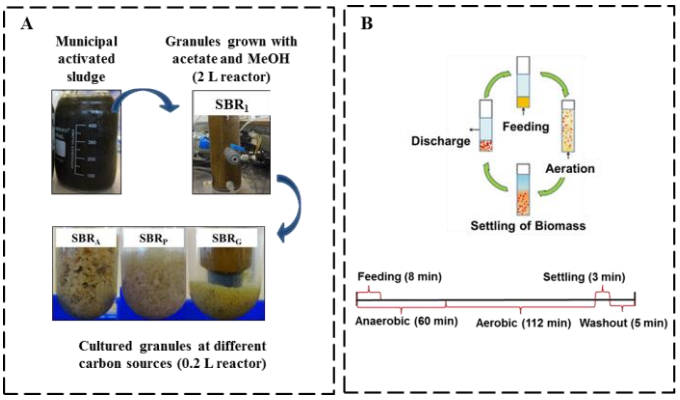

Figure 1. Representation of the granule formation. (A) Aerobic granules formed using activated sludge as a seed. (B) Schematic of the different steps in the sequential batch aerobic granular reactors used in this experiment.

2.1.2 CONVENTIONAL PARAMETERS

Total and volatile suspended solids (TSS, VSS), chemical oxygen demand (COD), nitrite, nitrate, phosphorus were determined according to standard methods (APHA, AWWA, WPCF 2005). Ammonium was determined using intelliCAL ammonium ISENH4181 probe from Hach Company and the HQ40d.

2.1.3 DNA EXTRACTION, PCR AMPLIFICATION OF 16S RRNA GENES AND SEQUENCE DATA PROCESSING.

Bacterial DNA extraction from 50 mg of aerobic granules was carried out using the `Phosphate, SDS, chloroform-bead beater` method (Miller *et al*., 1999). The hypervariable V4-V5 region of the 16S rRNA gene was targeted using primers. DNA was quantified using Qubit, with DNA quality and quantity measured using the agilent bioanalyzer 2100. Amplicon sequencing was performed using the MiSeq Illumina sequencing plataform. 16S rRNA gene sequences were processed using mothur (Schloss *et al*., 2009), UPRASE pipeline (Edgar, 2013), USEARCH_64 (Edgar, 2010), SILVA database, QUIME 1.9 (Caporaso *et al.*, 2010) and Greengenes (DeSantis *et al*., 2006).

2.2 RESULTS AND DISCUSSION

Removal of carbon, ammonium and phosphorus was successfully done simultaneously for each single aerobic granular reactor under study. Aerobic granules made in SBR1, already in operation for 180 days, were used as an inoculum for SBR_A, SBR_P and SBR_G. Reactors SBR_A; SBR_P and SBR_G were gradually formed over a period of 20 days until stable biomass was formed and simultaneous nutrient removal was achieved. As can be seen in Figure 2.A, carbon source affects the morphology of the granules. Removal of carbon, ammonium and phosphate were 86±6, 76±12 and 73±1 % respectively. Figure 2.B shows the removal of ammonium, phosphorus and carbon for each carbon source where it can be seen that carbon, ammonium and phosphorus are equally removed independently of the carbon source. Granular system provides high and stable rates of metabolism for the primary carbon substrate removals independently of the carbon source added.

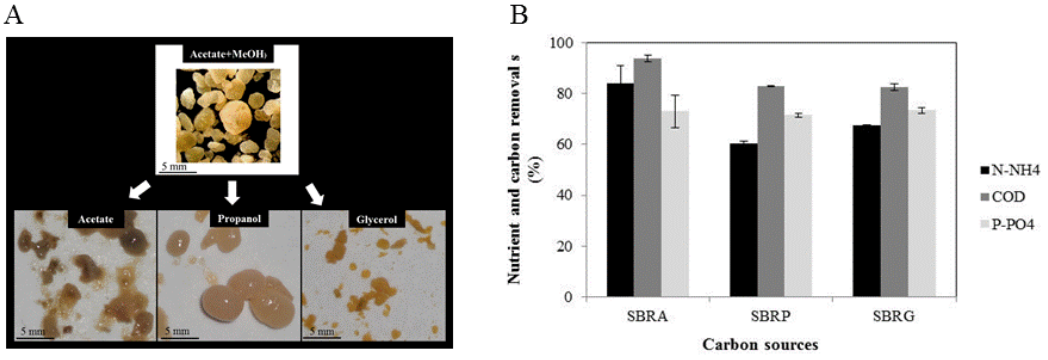

Figure 2. Performance of aerobic granules. (A) Morphology of granules growth under different carbon sources. (B) Average removal of ammonium, phosphorus and carbon done simultaneously by acetate, propanol and glycerol growth granules.

The majority of the bacterial 16S rRNA sequenced grouped with members of *Proteobacteria* (66% on average). The remaining predominant phylum is member of the *Bacteriodetes*, *Actinobacteria* and *Verrucomicrobia*. Richness at genus level was (OTUs): 4602, 3017 and 2595 for acetate, propanol and glycerol grown granules, respectively. Acetate is an easy to metabolize carbon source, thus most of the heterotrophs have the capability to obtain energy from it, however the metabolization of propanol and glycerol is more specific for a certain group of bacteria, leading to a decrement in the number of different taxonomic species in the reactor. Figure 3 shows a list of the genus level of the bacteria dominant in each system (>2%, and others which are bacteria without taxonomic assignment). Figure 3.A shows the bacteria at genus level. Dominant bacteria at genus level were different depending on the carbon source used as a feed. *Prostecobacter* and *Chryseobacterium* dominated the granule growth with acetate; Zoogleae dominated the reactor feed with propanol and *Citrobacter*, *Microbacterium and Rhodococcus* dominated the granule feed with glycerol. Citrobacter and Rodococcus are well known bacteria used for bioremediation, especially Rodococcus that have a notable metabolic versatility. Each bacterium possess different pathways to metabolize a carbon sources, each pathway ends in the degradation of the carbon sources- efficiency of COD removals, however every pathway generates its own specific metabolites and enzymes of each system conditions.

A

	Reactors and bacterial abundance (%)		
Main bacterial genus	SBRA	SBRP	SBRG
Citrobacter	0.0	0.0	**27.0**
Microbacterium	0.0	0.0	**22.6**
Rhodococcus	0.0	0.0	**9.1**
Propionicimonas	0.0	0.0	**1.9**
Zoogloea	**1.1**	**80.2**	**1.2**
Prosthecobacter	**18.8**	0.0	0.0
Chryseobacterium	**14.1**	**1.2**	0.0
Methylobacillus	**6.3**	0.0	0.0
Paludibacter	**6.0**	0.0	0.0
Rhodobacter	**2.8**	0.0	0.0
Brevundimonas	**2.7**	0.0	0.0
Fluviicola	**2.3**	0.0	0.0
Acidovorax	**1.8**	**1.3**	0.0
Sediminibacterium	**1.7**	0.0	0.0
PSB-M-3	**1.2**	0.0	0.0
Pseudomonas	**1.2**	1.0	0.0
Blvii28	**1.1**	0.0	0.0
Cloacibacterium	**1.1**	0.0	0.0
Stenotrophomonas	**1.1**	0.0	0.0
Paracoccus	0.0	0.0	0.0
Delftia	0.0	0.0	0.0
Acinetobacter	0.0	0.0	0.0
Others and < 2%	**36.8**	**16.3**	**38.2**

B

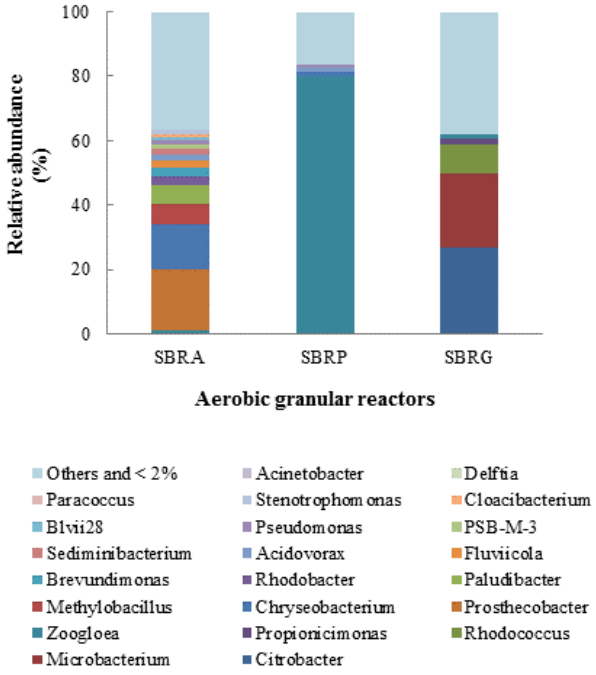

Figure 3. Summary of the bacterial genus identified by 16S rRNA analysis of each reactor. (A). Table with the specific values. (B). graph representation for each bacteria.

3 SHOCK WITH OMPS.

3.1 METHODS

Twenty millilitres of effluent previously centrifuged- 15,000 rpm for 20 minutes at -4°C, from each reactor, were used to extract the OMPs by solid phase extraction (SPE). Analytes were extracted using 6 cc Oasis HLB cartridges (Oasis) following the method described in (Vanderford *et al*., 2003). Quantification of analytes was done via LC-MS analysis using a Shimadzu 2020 Series LC-MS (Shimadzu, Japan) equipment with an Agilent ZORBAX Eclipse Plus C18 column (Agilent Technologies, Germany) following the method described in (Ferrer *et al*., 2008).

3.1.1 EXPERIMENTAL SET UP AND REACTOR OPERATIONS

Biotransformation of pharmaceuticals by granules growth with acetate, propanol and glycerol was determined by the quantification of the removal in each system as well as in autoclaved medium and biomass. Reactors were adjusted to have a concentration of 1 g l^{-1} VSS^{-1} and an initial concentration of 125 $ugl^{-1}vss^{-1}$ per reactor and operated under the same condition described in section 2.2.1. Autoclaved synthetic feed and granules were used as control experiments were abiotic removal was quantified.

3.2 RESULTS AND DISCUSSION

For control experiments, abiotic removal was (ug l^{-1} VSS^{-1}): 6±3, 3±3, 2±2 and 8±2 for IB, CBZ, TMP and SMX respectively. NPX and TYL, did not show any abiotic removal. All the reactors-independently of the carbon source supplied, were able to biotransform the pharmaceuticals added to each reactor. However the efficiency was different depending on carbon sources supplied. The average biotransformation for the bulk of micropollutants was (ug $l^{-1}VSS^{-1}$): 8 ± 5, 30 ± 16 and 65 ± 38 for AGSs provided with acetate, propanol and glycerol, respectively. Tylosin biotransformation was quite different, with (ug l-1VSS-1) 87, 64 and 38 for acetate, propanol and glycerol as the respective primary carbon source. Figure 4.A shows the biotransformation of each compound per gram of granules, where clearly an increase in carbon complexity increases pharmaceutical biotransformations. Also, figure 4.B shows an association of pharmaceutical biotransformation by categories, that antinflamatories have the greatest biotransformation as well as the most directly correlated with the richness of the bacterial community. Thus, microorganisms that are more adapt to degrade hard compounds could be capable to biotransform pharmaceuticals by the production of specific enzymes that can react with the compounds to be removed from the system. As seen before, increases in carbon complexity reduce the richness of the granular systems, and therefore, as we increase carbon complexity, the bacteria are unlikely to have more functional traits for pharmaceutical removals. However, this result shows that richness is not a key aspect for the pharmaceutical removals.

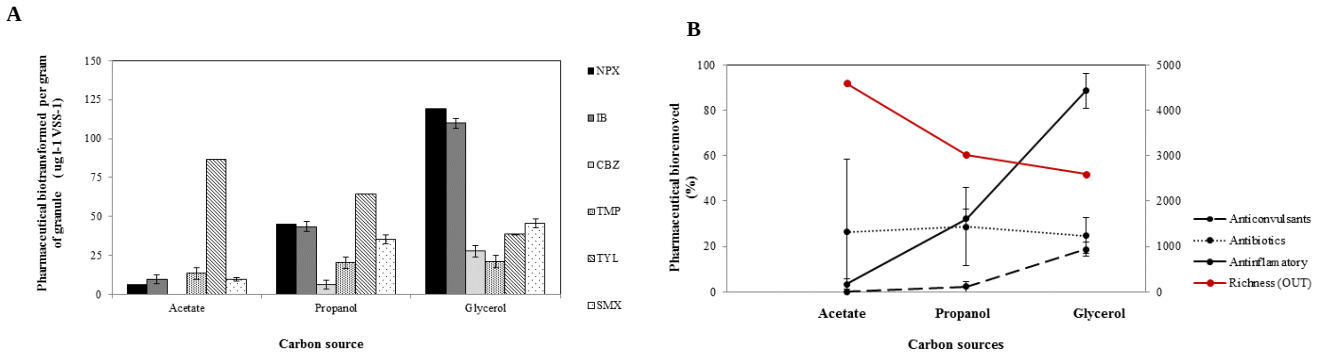

Figure 4. Biotransformation of pharmaceuticals for each carbon source. (A). Biotransformation for each micropollutant. (B). Biotransformation for pharmaceutical category.

CONCLUSIONS

Aerobic granules can remove ibuprofen, naproxen, sulfamethoxaxole, trimethoprim, tylosin and carbamazepine from wastewater. The use of more complex carbon sources as granule primarily substrate enhances biotransformation of the majority of the studied OMPs (expect tylosin) without impacting the nutrient and carbon removal.

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