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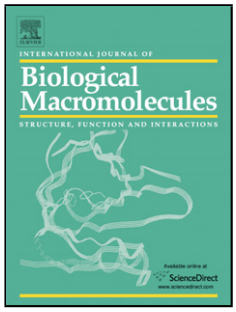

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Fractionation of microbial populations in a PHA accumulating mixed culture and associated PHA content and composition

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Abstract

The uniformity of PHA composition and content across groups of organisms in mixed cultures was considered. An activated sludge microbial community, with an average PHA content of 20 wt%, was fractioned by Percoll assisted buoyant density separation. The microbial community in the two principal fractions was characterised using amplicon pyrosequencing. While organisms were common to both fractions, the relative abundances of species were found to be different between the two fractions. The average PHA content in one of the fractions was found to be higher (24 wt%) than the other (16 wt%); separation was considered to be in part driven by the density difference associated with PHA content, but also by other factors such as cell dimension and cellular morphology. But while differences in PHA content were observed, the PHA composition in both fractions was found to be approximately the same (43 – 44 mol% HV), which shows that distinct groups of microbial populations within mixed cultures may generate PHA with similar average copolymer composition.

Keywords

Polyhydroxyalkanoates; PHA; Mixed cultures; Buoyant density gradient centrifugation; pyrosequencing.

1. Introduction

Polyhydroxyalkanoates (PHAs) have drawn increasing attention because of their biodegradability, biocompatibility and potential for production from renewable resources [1]. More than 300 species of Gram-positive and Gram-negative bacteria as well as a wide range of archaea synthesize PHA as intracellular insoluble cytoplasmic inclusion bodies in the presence of excess carbon source and limited amounts of nutrients such as phosphorus or nitrogen. The production and material properties of PHA have been widely reviewed, e.g.Laycock et al. [2]. The use of mixed cultures, potentially derived from activated sludge used in the treatment of wastewater, might offer a cost effective approach to PHA synthesis as it avoids requirement for sterilisation and allows for the use of fermented residuals, industrial process waters and wastes as feedstocks [3, 4].

Many biologically produced PHAs, from both pure and mixed cultures, have a broad compositional distribution and are in fact blends of copolymers of differing hydroxyvalerate (HV) and hydroxybutyrate (HB) content rather than a single copolymer of narrow composition [5, 6]. It is known that PHBV copolymers in blends will at least partially phase separate if the difference in HV content between components is more than 12% [7]. Therefore the macro-scale architecture, and consequently the mechanical properties, of such blends are anticipated to be influenced by the polymer make-up.

Fractionation studies show that commercial PHA materials from pure cultures can be a mixture of random copolymers [8, 9]; it is expected that this co-polymer blend nature of products from mixed cultures, enriched on mixed feeds, will be similarly evident. In the presence of multiple substrates, microbial populations specialised in those different substrates are selected, coexisting in the reactor by adapting to different survival niches [10]. The

structure, physio-chemical properties, monomer composition and the number and size of the PHA granules apparently vary depending on the organism and the carbon source utilized by the microorganisms [11]. So it is possible that populations with distinct substrate affinities will produce different co-polymers and consequently blends of PHA copolymers will result during the polymer recovery from a mixed-culture biomass. However, the reason for broad copolymer compositional distributions in both pure and mixed cultures is likely to be more complex than this, being potentially based on intracellular differences with respect to substrate utilisation within the same bacterial family. Overall, the origin of complex monomer distribution in bacterial copolyesters has not yet become clear in either pure or mixed cultures, but from these fractionation studies it can be understood that it is of interest to gain more fundamental understanding of the inter- and intra-cellular nature of the co-polymer distribution and thereby identify the principal source of variability in PHA co-polymer distribution that have been identified in PHA recovered from mixed culture biomass.

One method to follow specific groups of populations in mixed cultures is by isolating each bacterial species (based on tagging or by flow fractionation etc.), followed by pure culture cultivation. However, this approach may not be effectively achieved for the microbial community in activated sludge obtained from wastewater treatment plants (WWTPs) because only small quantities of individual species would be obtainable and many of the microorganisms are anticipated to be non-culturable under laboratory conditions [12-14]. So, methods that rely on selective separation of the biomass are required to discriminate distinct population fractions. Buoyant density-gradient centrifugation is a useful technique for separating cells or organelles of different densities. It has even been trialled as a method to concentrate PHA rich biomass in mixed cultures: Oshiki et al. [15, 16] concentrated the number of PHA rich cells in mixed cultures through density gradient centrifugation. In one study, the authors considered two mixed cultures, each with about 20% of cells being rich in PHA [16]. After density gradient centrifugation (using a Percoll solution of 1.087 g mL^{-1}) they obtained pellets with 50-60% of cells being rich in PHA. The approach was based on

the separation principle that PHA granules have a higher density of 1.15 to 1.25 g mL^{-1} [17] than activated sludge at 1.02 to 1.06 g mL^{-1} [18].

In this study we aimed to separate a PHA accumulating mixed cultures by buoyant density gradient centrifugation and thereby produce distinct microbial fractions. The work builds on that presented by Oshiki et al. by examining the microbial community as well as the PHA content and composition within the respective fractions. These were analysed with a view to gaining a deepened understanding of the homogeneity or heterogeneity of distribution for PHA within a PHA-rich mixed culture biomass.

2. Materials and Methods

2.1 Materials

Acetic acid and propionic acid were obtained from Sigma Aldrich and were of 98% purity. Chloroform was of HPLC grade (99.9% purity) and was obtained from Sigma Aldrich. Percoll consists of colloidal silica particles of 15-30 nm diameters (23 wt% in water) that have been coated with polyvinylpyrrolidone (PVP). Percoll was obtained from Sigma Aldrich with a density of 1.130 +/- 0.005 g mL^{-1}. All other chemicals were obtained from Sigma Aldrich and were of at least 98% purity.

2.2 PHA accumulation and chemical analysis

Waste Activated Sludge (WAS) was obtained from Luggage Point Wastewater treatment plant (WWTP), Queensland, Australia, and was kept at 4 ^{o}C before being warmed up to room temperature before use (21–25 ^{o}C) and aerated until reaching stable dissolved oxygen concentrations close to saturation. PHA was accumulated in this WAS without enrichment of the population [3], using a pulse feeding sequence with the feed consisting of a solution containing acetic acid (35.71 g L^{-1}) and propionic acid (6.67 g L^{-1}), with a chemical oxygen demand (COD) ratio of 80:20 acetic acid: propionic acid. Accumulation was undertaken under aerobic conditions at room temperature for 8 h in a laboratory-scale batch reactor with a working volume of 2 L. Air was supplied by an air pump at the rate of 2 vvm (L L^{-1} min^{-1}). pH was monitored but not controlled. Experiments were managed using the Opto 22 PLC controlled program based on dissolved oxygen (DO), and feeding was based on the use of a DO control point set at 1.8 mg L^{-1} to initiate the addition of the carbon source following depletion of the previous dose of feed. DO returning to the set point (1.8 mg L^{-1}) indicated that readily available substrate had been taken up by the biomass and so the next dose of substrate (5 mL at 50 g L^{-1}) was pumped in automatically. This pulse-wise feeding strategy was used because it has been observed that PHA accumulation is enhanced when the carbon source is added in a number of controlled pulses rather than in a single, larger pulse [19-21].

During the course of the accumulation, grab samples were taken at selected time points between 0 to 8 h for Gas Chromatography (GC), Volatile Fatty Acids (VFA), Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) analysis. For the

measurements of NH_4-N and phosphate phosphorus (PO_4-P) concentrations, a flow injection analyser (FIA) was (Lachat QuickChem8000). Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were measured gravimetrically by drying a filtered and washed, known volume in a 100 °C oven overnight (for TSS), and for 2 h in a 550 °C furnace (for VSS).

PHA was measured by GC analysis: 10 mL samples were prepared according to the hot chloroform method [22], with some modifications [23]. 10 mL samples were taken, with 10 drops of 40% formaldehyde added to stop all activity. The samples were centrifuged at 3750 rpm (3267 x g) for 5 mins. The supernatant was removed and the pellet dried in an oven at 110 °C for 24 h (overnight). Next, 2 mL of acidified methanol with benzoic acid as internal standard (3 wt% of H_2SO_4) and 2 mL of chloroform were added followed by digestion of the sample for 20 h at 100 °C. After cooling, 1 mL of milli-Q water was added to promote methanolysis. After 1 h settling, the organic phase was transferred to a vial and 3Å molecular sieves added (to remove water) in preparation for GC analysis. 1 mL of the chloroform phase (bottom phase) was then removed for analysis on a Perkin-Elmer gas chromatograph (GC). 3 µL of this chloroform phase was then analysed using a flame ionisation detector (FID) at 300 °C and a DB-5 nonpolar capillary column (30 m × 0.25 mm × 0.25 µm) at a range of 80-270 °C. Biologically sourced PHBV copolymer (70 mol% HB) (Sigma Chemicals, USA) was used as a standard.

VFAs were determined by means of a Perkin-Elmer GC. The column was a DB-FFAP 15 m × 0.53 mm × 1.0 µm (length ID film) at 140 °C, while the injector and flame ionisation detector (FID) were operated at 220 °C and 250 °C, respectively. High purity helium was used as a carrier gas at a flow rate of 17 mL min^{-1}.

2.3 Buoyant density separation of PHA accumulating cells

Mixed microbial cells containing PHA were harvested by centrifugation at 3750 rpm (3267 × g) for 10 min (wet biomass) using a Beckman SX4750 rotor in an Allegra X-12 Series centrifuge. To the wet biomass, 25 mL distilled water was added to make a cell suspension for separation studies. Percoll was used for density gradient centrifugation because of its nontoxicity, low viscosity, ease of preparation at the desired osmolarity and pH [24]. The cell

suspension (9.6 mL) was layered on top of a 40% Percoll gradient working solution that contained 10.8 mL of Percoll, 1.2 mL of 2 M NaCl, and 14.4 mL of distilled water. The mixture was centrifuged for 10 min at 3750 rpm (3267 x g). In the same fashion, different selected concentrations of Percoll ranging from 10% to 45% were prepared.

2.4 Quantitative determination of PHA in the separated cells

PHA content and composition of the cells separated by buoyant density gradient were analysed through the following procedure. The samples were centrifuged, again at 3750 rpm (3267 x g) for 10 min, and the pellet and the float were pipetted out in to fresh new tube. Both were then washed with milliQ water, re-centrifuged, and the supernatant decanted. The separated cells (pellet and float) were washed repeatedly to effectively rinse off all the Percoll. All samples were then dried at 100 °C overnight. Approximately 20 mg of separated cells were added to 2 mL of chloroform and 2 mL of an acidified methanol solution containing 100 mg L^{-1} of sodium benzoate as an internal standard were added. The samples were heated to 100 °C for 20 h and the GC analysis performed as above.

2.5 Microscopic analysis of the separated cells

Samples were divided into two fractions (pellet and float) after buoyant density gradient centrifugation, with the middle section not being analysed due to it being too disperse. To view PHA inclusion bodies, samples were heat fixed, stained with 1% (wt vol^{-1}) Nile blue A (NBA) (Sigma, NSW, Australia) for 15 min at 55 °C, destained for 30 s in 8% (vol%) acetic acid, water washed, air dried, and viewed through an Olympus BX51 fluorescent microscope equipped with a CCD DP70 (Olympus, Japan) camera. To determine the overall abundance of PHA-accumulating microorganisms in the cells, dual chemical staining with NBA and DAPI was performed. Because PHA granules emit strong fluorescence when they are bound with NBA, PHA-accumulating cells are easily identified by fluorescence microscopy staining with NBA [25].

2.6 Microbial characterization of the separated cells

The microbial characterization of the separated cells was accomplished through pyrosequencing. DNA was extracted from the control and the two fractions after buoyant density separation using the Q-Biogene Fast DNA SPIN kit for soil (MP Biomedicals, Seven Hills, NSW, Australia) according to the manufacturer's instructions. DNA integrity was

checked by agarose gel electrophoresis in 1% agarose gels and quantified using a Nanodrop™ ND1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). DNA extracted from separated cells was amplified through PCR. PCR amplification of the 16S rDNA was performed using the eubacterial primers 926f and 1392r. The PCR mixture consisted of 1 μL of DNA template, 1 μL of each primer 926f (5'-AAACTYAAAKGAATTGACGG-3') and 1392r (5'-ACGGGCGGTGT-GTAC-3') [26], 5 μL of 10X PCR buffer (Applied Biosystems, VIC, Australia), 1 μL each of a 200 μM solution of dATP, dCTP, dGTP, and dTTP, and 34.5 μL of sterile water. Each reaction mixture was overlaid with filter-sterilized mineral oil. The reaction mixtures were heated to 100 °C, followed by the addition of 0.5 μL of AmpliTaq Gold Taq polymerase (5 U μL^{-1}; Applied Biosystems, VIC, Australia.). The PCR was conducted on a MyCycler Thermal Cycler System (Bio-Rad Laboratories Inc., Hercules, CA, USA), with a programme comprising an initial 5-min denaturation step at 95 °C, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 mins, and a final 10-min extension step at 72 °C [27]. Equal volumes of triplicate PCR products were pooled and unincorporated primers and reaction components were removed using a Qiaquick PCR purification kit (Qiagen, Australia) according to the manufacturer's instructions and the purified products were resuspended in DNase-free water. For each set of PCR amplifications, a control reaction without template was performed to check the kit and solution purity. Amplicons were pooled in equimolar concentrations and sequenced using a 454 GS FLX Titanium sequencer (Roche) as per the manufacturer's protocol. Pyrosequencing results were analysed through a local implementation of the ACE Pyrosequencing Pipeline (https://github.com/Ecogenomics/APP) in which sequence reads were split according to the barcode in QIIME [28]. De-multiplexed sequences were then trimmed to 250 bp length and de-noised by ACACIA [29], a tool for homopolymer error-correction that has greater scalability than existing tools and is used to maintain sensitivity without compromising genuine signal in the data [30]. Sequences with 97% similarity were assigned to operational taxonomic units (OTUs) by CD-HIT-OTU [31] and aligned by *Pynast* [28]. Each sequence was then assigned to the taxonomy with BlastTaxonAssigner in QIIME through greengenes database (2013 Aug release). Finally the non-normalized OTUs table and rarefaction curve were generated by QIIME. Normaliser (https://github.com/minillinim/Normaliser) was used to find a centroid normalised OTUs table with 2300 reads per sample. The sequences recovered from the dominant 10 OTUs were also compared with other sequences previously deposited in GenBank

(http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) and genus level classification were assigned (if >99% identity were obtained). The normalised OTUs table was then summarised to phylum level and used to predict the variance of microbial community. Further, the normalised OTUs table was imported to R (version 2.8.1). After a log transformation, a heat map was drawn up including the top 30 abundant OTUs. OTUs were clustered based on Euclidean distance in the heat map.

3. Results and Discussion

3.1 PHA accumulation

PHA was accumulated in non-enriched waste activated sludge. The substrate for accumulation was a mix of acetic and propionic acids (80:20 on a gCOD basis). PHA accumulation was run for 8 hours. After this time, consumption of further carbon and hence PHA accumulation effectively ceased and a PHA content of 20 wt% (gPHA $gTSS^{-1}$), and composition of 57 mol% HB – 43 mol% HV was obtained.

3.2 Biomass fractionation: PHA content and composition

Percoll solutions with densities of 1.013, 1.025, 1.037, 1.049 and 1.055 g mL^{-1} based on 10%, 20%, 30%, 40% and 45% dilute Percoll solutions respectively were trialled in this study, with the 1.049 g mL^{-1} (40%) solution being selected for full fractionation as it delivered the most distinct fractions.

The material balance showing the fractionation in terms of PHA and solids in the 40% Percoll separated samples is shown in Table 1. After buoyant density separation a fraction of the biomass settled at the bottom of the centrifuge tube as a pellet (50%), a fraction floated (30%) and a residual remained in suspension (20%). Two distinct fractions were considered for analysis: the pellet and the float. Table 1 shows that the PHA content of the pellet was higher than of the float.

[TABLE 1 near here]

The PHA content observed in the pellet and float was 24 wt% and 16 wt%, respectively. Considering that PHA is denser than activated sludge, separation was likely in part driven by

the density difference associated with PHA content. Density gradients have been applied to collect PHA rich cells from cultures containing cells with varying PHA content [16]. However, while Oshiki et al. [16] managed to concentrate the number of PHA rich cells using density gradient centrifugation, the authors found that PHA recovery in the pellet was actually reduced as the density of the solution was increased. The authors recovered less than 20% of PHA in the pellet when the optimal density of 1.087 g mL^{-1} was used. They attributed this to the aggregation of PHA rich and sparse cells; an additional factor may have been inconsistencies between the concentration of PHA rich cells and mass fractions. The results of the present investigation and associated unpublished activities also suggest that the density gradient separation methods are not universally applicable to industrial processes for recovering PHA rich biomass. In this work, separation was not based on PHA alone, evidenced by the fact that in low concentration Percoll solutions (10%, 20% and 30%) most of the sludge material settled, indicating, based on assumed densities of PHA and activated sludge, that most of the biomass had a PHA content of greater than approximately 15%. At 40% Percoll solution, effective separation was achieved and higher PHA content in the pellet and lower in the float was observed. But in high Percoll solution (45%) all the sludge material floated, erroneously indicating, based on assumed densities of PHA and activated sludge, that none of the biomass had greater than approximately 20% PHA content; GC analysis showed that it did. By contrast, in an associated unpublished experiment using biomass with a PHA content of 56 wt%, separation occurred at a Percoll concentration of only 25%, and the float layers (74% of separated biomass) had elevated PHA content (63.30 ± 0.01 wt%, n=8) compared to the pellet (6% of biomass, which had a PHA content of 56 wt%, n=1). These results highlight that factors additional to PHA content may contribute to fractionation in a density gradient separation. In mixed culture biotechnology, density separation has been applied to separate microbial populations, for example, for differentiation of methanotrophic bacteria [32], separation of bacteria from freshwater sediments [33], separation of subpopulations with different gene expression patterns [34], separation of cells of different viability or activity [35], separation of respiring bacterial cells from mixed populations in natural seawater [36], and separation of polyphosphate-accumulating cells [37]. However, in the present investigation we find that PHA content is not a sufficiently dominating attribute of the biomass to control fractionation in density gradient separation when using a relatively low speed centrifuge, although it would be worth exploring if separation would be more effective if ultracentrifugation is used.

The monomer composition in pellet and float were effectively the same, being measured as P(3HB-*co*-44% 3HV) in the pellet and P(3HB-*co*-43% 3HV) in float. It has been demonstrated through fractionation studies that these copolymers are more than likely comprised of blends of P(3HB-*co*-3HV) of differing HV contents [38]. Originally this result was attributed to the difference in composition of PHA accumulated in different bacterial species present in the community. However, the fact that we have obtained very similar bulk compositions in two fractions that we later show (Section 3.3) to have differing community composition may point towards a more complex explanation, such as contribution of intracellular variation to distribution of co-polymer blends.

3.3 Microbial communities

Pyrosequencing recovered total 8380 raw reads, grouped into 362 OTUs from two fractions (pellet and float) and the original biomass. The errors in pyrosequencing reads have been estimated as approximately 100–250 base pairs and 5–10 errors kb^{-1} [39], which is not considered to be significant with respect to classification of phyla [40]. Figure 1 shows that the phyla of the pellet were similar to that of the float, but the relative abundances were different. *Proteobacteria* dominated both the pellet (52.3% of the total sequences) and the float (41.6%). In the pellet the remainder of the community consisted of *Bacteroidetes* (17.8%), *Chloroflexi* (9.8%), *Actinobacteria* (4.0%), *Firmicutes* (3.3%), *Planctomycetes* (2.4%), and *Acidobacteria* (1.1%), while in the float it consisted of *Chloroflexi* (18.8%), *Bacteroidetes* (12.4%), *Actinobacteria* (9.5%), *Firmicutes* (6.0%), *Planctomycetes* (2.4%), and *Acidobacteria* (1.4%).

[FIGURE 1 near here]

Figure 2 shows that at the OTUs level the differences are even more pronounced, with the community of the pellet being more closely aligned with the original than it was to the float. The heat map shows *Candidatus Competibacter phosphatis* was the most abundant OTU present, representing 28.3% of all classified sequences in the pellet and 17.7% in the float. *Candidatus Competibacter phosphatis* is a non-culturable glycogen accumulating organism of the class *Gammaproeobacteria*, with all phylotypes having the ability to anaerobically store volatile fatty acids (VFAs) as PHA without observed polyphosphate cycling. These

organisms are commonly found during post-FISH chemical staining for PHA in full-scale EBPR wastewater treatment plants [41]. The distribution of other OTUs, including *Actinomycetales* and *Dokdonella*, varied substantially in relative magnitude. These organisms were 50 to 100% more prevalent in the float (4.1%, 4.5%) than the pellet (2.4%, 2.7%).

[FIGURE 2 near here]

3.4 Microscopy

Microscopic investigation with staining using Nile blue A and DAPI was undertaken to confirm the presence of PHA in the fractions. The cells in the pellet contained PHA granules whereas the float possessed some cells apparently not containing any granules (data not shown).

4 Conclusions

In this work a mixed culture population with PHA content of 20 wt% and copolymer composition of 43 mol% HV was separated into two fractions (pellet and float) containing distinctions in relative abundances of microbial populations. The pellet and float possessed different PHA content, but density separation was apparently not due solely to PHA content showing that buoyant density separation is not necessarily effective for concentrating PHA rich cells as has been previously been suggested in the literature. Observed differences in net PHA content could be a result of the measured differences in the microbial communities or simply differences in stages of the growth and accumulation cycles within individual communities. The fact that PHA composition in both fractions was found to be similar (43 – 44 mol% HV) indicates the latter might be most likely, but shows that groups of microbial populations within mixed cultures do not necessarily generate PHA with unique composition, although at this low level of PHA content the degree of separation may not be enough to discriminate significant differences.

5 Acknowledgements

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Table 1. Buoyant Density separations showing different PHA content and monomer composition in the pellet and float for 40% Percoll separated samples

Fractions	PHA Content* (wt% ±s.d.)	TSS (g)	PHA Composition* (mol% ± s.d.)	
			HB	HV
Control[1]	19.8±0.2	0.101	57.5 ±0.7	43.5±0.7
Pellet	23.8±0.6	0.053	56.5±0.7	44.5±0.7
Float	16.1±4.9	0.037	57.5±2.1	43.5±2.1

[1] Control: 0% Percoll solution, same wash procedure as others

* duplicate samples for PHA content and composition

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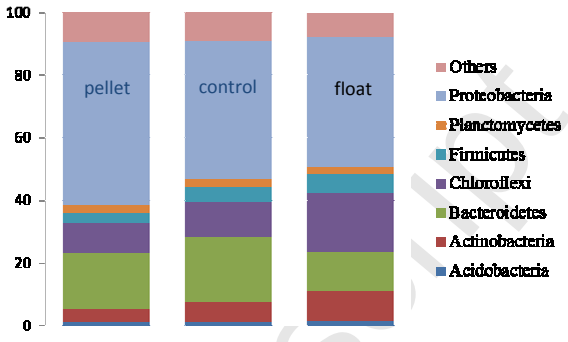
Bacteria Phyla	Community Composition (%)		
	Control	Pellet	Float
Acidobacteria	1.3	1.1	1.4
Actinobacteria	6.0	4.0	9.5
Bacteroidetes	20.9	17.8	12.5
Chloroflexi	11.2	9.8	18.8
Firmicutes	4.9	3.3	6.0
Planctomycetes	2.6	2.4	2.4
Proteobacteria	44.0	52.3	41.6
Others	9.1	9.5	7.7



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495 Figure 1. Phylum level microbial community of pellet and float sample separated from 40%
496 Percoll and control from 0% Percoll.

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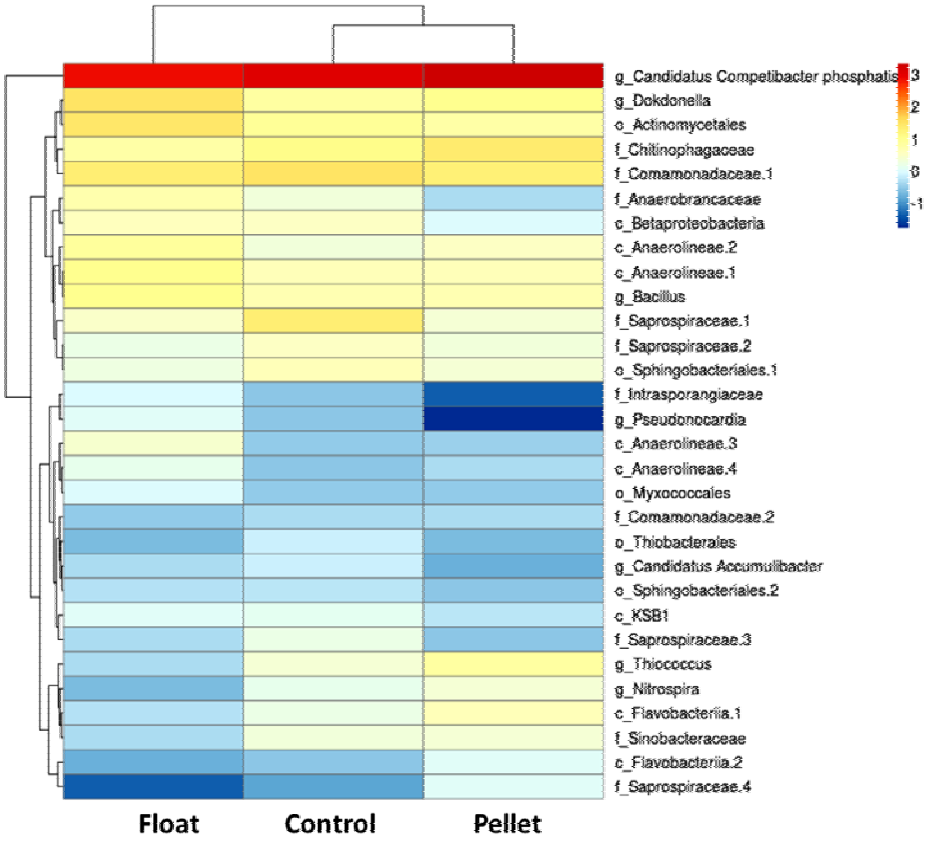

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503 Figure 2. Heat map for relative abundances (after transformation) of top 30 abundant OTUs.

504 Each OTU is labelled to the lowest known classification (g: genus, f: family, o: Order, c:

505 class) from greengenes (or genebank) database. Clusters based on Euclidean distance.

mixed culture

buoyant density separation

FLOAT

sub-community 1

16 wt% PHA

57% HB

PELLET

sub-community 2

24 wt% PHA

56% HB