**Materials and Methods**

**DNA and RNA extraction, PCR and high-throughput amplicon sequencing**

Total RNA and DNA were extracted from 5 MAIFAS (micro-algae seeded reactor) and 3 IFAS (control reactor) samples as previously described (Pitkänen et al., 2013) with some minor modifications. Briefly, the AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Hilden, Germany) was used to extract total nucleic acid. RNA was further purified using Ambion TURBO DNA-free DNase kit (Life Technologies, Grand Island, NY). The concentration and purity of RNA and DNA were determined using the Qubit 2.0 Fluorometer with Qubit RNA and dsDNA HS assay kits, respectively (Life Technologies). cDNA was generated using random hexamer primed Superscript III system for RT-PCR (Life Technologies). Samples (cDNA and DNA) were stored at -20°C until used for next generation sequencing. The cDNA and DNA were used as templates to generate independent libraries targeting bacterial 16S ribosomal RNA genes (rDNA) and transcripts (rRNA). We used barcoded 16S rRNA gene targeting primers (i.e., 515F and 806R) described in Caporaso et al. (2011) and sequenced the targeted product (i.e., 291 bp) in both directions using an Illumina MiSeq PE250 sequencing kit. Sequencing was performed at the Cincinnati Children’s Hospital Medical DNA Sequencing and Genotyping Core facility.

**Next generation sequencing data preprocessing and analysis**

Sequence reads (16S rDNA- and 16S rRNA-based) were processed and analyzed using Mothur software (Schloss et al., 2009). Sequence reads that did not fit the following criteria were discarded from further analyses: did not form contigs, deviated considerably from the expected PCR size product, had ambiguous bases, and had homopolymers greater than 8 bases long. Sequence reads were grouped at a 97 % similarity and the consensus sequences were then identified using Mothur and the Silva (Quast et al., 2013) database as a reference. Prior to the classification analysis, a prescreening step was performed with a randomly selected subset of all the sequences generated per sample (n=10,000) to further filter out chimeras and difficult to align sequences. Excel was used to determine the overall relative abundance of representative sequences at different taxonomic levels (e.g., class, order, family, genus). Sequences were analyzed using Blast (<http://www.ncbi.nlm.nih.gov/BLAST/>) and RDP classifier (Wang et al., 2007) to further confirm their phylogenetic affiliation and to classify sequences at a low taxonomic level (genus and species) whenever possible.

**Results and Discussion**

A total of 70,525 and 69,592 sequences were analyzed from the rDNA and rRNA libraries and used to describe the bacterial composition and identity of metabolically active bacteria within the reactors (supplemental Table 1). The sequencing data suggested that there are some similarities regarding the bacterial composition between the reactors. Each reactor included members of bacterial phyla such as Protoebacteria (e.g., alpha-, beta- and gamma-proteobacteria), Bacteroidetes (Cytophagia, Flavobacteriia, Saprospirae), Nitrospirae (*Nitrospira*), and Acidobacteria (*Chloracidobacterium*)(Table 1). However, there were some striking differences in relative abundance between reactor type. For example, members of the beta-proteobacteria were among the most abundant groups in both reactors but some of the species were more prevalent in one reactor type. Specifically, *Candidatus* Accumulibacter was a numerically dominant in the MAIFAS reactor but barely detected in the IFAS reactor. In contrast, *Dechloromonas* represented >5% of the sequences in the control reactor versus < 0.4% in the seeded reactor. A similar result was observed for *Acinetobacter* (gamma-proteobacteria) as far as its relative abundance in the IFAS reactor was concerned.

Differences in relative abundance of several bacterial groups were also noted when sequencing library type results were compared. In the MAIFAS reactor, *Candidatus* Accumulibacter were more abundant in the rRNA (55%) than in the rDNA sequencing library (< 1%). In both reactors *Comamonadaceae*- and *Nitrosomonadaceae*-like sequences were more abundant in the rRNA sequencing libraries. In the IFAS reactor *Acinetobacter* was more than two times as abundant in the rRNA library than in the rDNA library. In other cases, the abundance of a bacterial group decreased in the rRNA libraries. This was the case for *Aeromonas*, *Lysobacter*, *Nitrospira*, Flavobacteriia, and Saprospirae. Sequences related to members of the phylum *Caldithrix* were greater in the MAIFAS rRNA and totally absent in the IFAS reactor. The presence of this bacterial group is intriguing as it has primarily been associated with hydrothermal sediments and considered to be nitrate reducing bacteria and obligately anaerobic (Miroshnichenko et al., 2003). Although its function in the MAIFAS reactor is unknown at this point, genome sequencing analysis of *C.* abyssi has reveled that carbohydrates such as starch, cellobiose, glucomannan and xyloglucan several can support its growth (Kublanov et al., 2017).

As rRNA transcripts are associated with protein synthesis, rRNA-based data have been used as a proxy for assessing the relative activity levels in several aquatic matrices (Pitkänen et al., 2013; Revetta et al., 2011). Moreover, shifts in rRNA:rDNA ratios may signal overall changes in relative metabolic activity in a given bacterial group (Kapoor et al., 2015a, b). Using this rationale, our data suggest that some groups are not only present in the reactor but more metabolically active than other groups. For example, rRNA:rDNA ratios suggest that *Nitrosomonadaceae* may be more actively involved in nitrogen removal than *Nitrospira*, particularly in the MAIFAS reactor. However, the fact that both nitrifying bacterial groups are present suggests that they might be occupying different ecological niches within these reactors. Moreover, several *Nitrosomonas* species were identified (e.g., *N. communis*, *N. europaea*, *N. oligotropha*, *N. ureae*), suggesting that ammonia removal is conducted by multiple populations. Additionally, the lower rRNA:rDNA ratios of several members of Bacteroidetes suggest that they are not removing organic carbon at a high rate as implied by their abundances in the rDNA libraries. Low metabolic activity of Bacteroidetes have also been observed in wastewater nitrifying enrichments (Kapoor et al., 2016).

Also notable is the fact that *Candidatus* Accumulibacter-like sequences were relatively rare in the IFAS reactor and over six times more abundant in the rRNA libraries that in the rDNA libraries in the MAIFAS reactor, suggesting that this bacterial group may be actively playing a role in phosphate removal in the seeded reactors. Previous studies have reported *Candidatus* Accumulibacter phosphatis as a dominant member of enhanced biological phosphorus removal (ESBR) sludge microbial communities (Flowers et al., 2013). In fact, *Ca.* Accumulibacteris capable of phosphorus removal in wastewater enrichments employing different oxygenic conditions (Camejo et al., 2016). *Ca.* Accumulibacter has previously been shown to inhabit microalgae-IFAS biofilms, which was also evident in our study. Possibly also related to phosphate removal were the dynamics of *Dechloromonas* spp. which relative abundance significantly decreased in the MAIFAS samples. Members of this genus have been shown to accumulate polyphosphate. As other potential polyphosphate accumulating bacteria were not detected or were present in very low numbers, the results of this study implicate *Ca.* Accumulibacter and *Dechloromonas* as the primary phosphate removing bacteria in the MAIFAS and IFAS, respectively, although the role of difficult to classify beta-proteobacteria cannot be discarded. Thus, our data strongly suggest that there may be biochemical interactions between the microalgae and different bacterial groups that promote the enrichment, and furthermore, stimulate the metabolic activity of *Ca.* Accumulibacter, resulting in increase of phosphate removal.

In conclusion, the addition of microalgae to the IFAS system promoted some significant changes in the bacterial community structure and the metabolic activity of several bacterial groups. Our data shows that sequencing analysis of rRNA and rDNA libraries provides a more robust picture of the microbial dynamics and potential biological interactions in microbial reactor studies.

TABLE 1. Distribution of bacterial 16S rRNA and 16S rDNA

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Class | Genus | MAIFAS-Mean | | IFAS-Mean | |
|  |  | RNA  (n=8584) | DNA  (n=8570) | RNA  (n=8714) | DNA  (n=9234) |
| Alpha-Proteobacteria | *Rhodobacteraceae\**  *Woodsholea* | -  14 | 18  127 (1.5%) | 33  93 (1.1%) | 145 (1.6%)  97 (1.1%) |
| Beta-Proteobacteria | *Candidatus* Accumulibacter  *Comamonadaceae\**  *Nitrosomonadaceae\**  *Nitrosomonas*  *Dechloromonas*  *Zoogloea*  Unclassified | 4719 (55%)  603 (7.0%)  126 (1.5%)  62  34  39  701 (8.2%) | 754 (8.8%)  192 (2.2%)  29  16  36  113 (1.3%)  620 (7.2%) | 64  904 (10%)  208 (2.4%)  70  477 (5.5%)  220 (2.5%)  3576 (41%) | 44  377 (4.1%)  30  14  565 (6.1%)  399 (4.3%)  2368 (26%) |
| Gamma-Proteobacteria | *Acinetobacter*  *Aeromonas*  *Lysobacter*  *Rheinheimera* | 20  -  -  - | 26  90 (1.1%)  45  - | 806 (9.3%)  17  62  42 | 379 (4.1%)  308 (3.3%)  110 (1.2%)  51 |
| Nitrospira | *Nitrospira* | 21 | 89 (1.0%) | 131 (1.5%) | 322 (3.5%) |
| Acidobacteria | *Chloracidobacterium* | 21 | 124 (1.5%) | 411 (4.7%) | 304 (3.3%) |
| Cyanobacteria\*\* | Unclassified | 21 | 50 | 29 | 15 |
| Cytophagia | *Cytophagaceae\**  Unclassified | -  - | 58  146 (1.7%) | -  80 | 64  428 (4.6%) |
| Flavobacteriia | *Flavobacterium*  *Cloacibacterium* | -  - | -  - | -  17 | 107 (1.2%)  191 (2.1%) |
| Phycisphaerae | Unclassified | 23 | 103 (1.2%) | 46 | 55 |
| Saprospirae | *Chitinophagaceae\**  *Saprospiraceae\** | -  72 | 683 (8.0%)  1558 (18%) | 21  - | 902 (9.8%)  59 |
| Unclassified | *Caldithrix* | 183 (2.1%) | 17 | - | - |

\* Family, \*\* Phylum, - less than 10 sequences

Supplemental TABLE 1. Distribution of bacterial 16S rRNA and 16S rDNA

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Class | Genus | R1 | | R2 | | R3 | | R4 | | R5 | | Mean (R1-R5) | |
|  |  | RNA  (n=8645) | DNA  (n=8215) | RNA  (n=8807) | DNA  (n=8645) | RNA  (n=8215) | DNA  (n=8807) | RNA  (n=8473) | DNA  (n=8473) | RNA  (n=8780) | DNA  (n=8712) | RNA  (n=8584) | DNA  (n=8570) |
| Alpha-Proteobacteria | *Rhodobacteraceae\**  *Woodsholea* | -  10 | 14  106 | -  12 | 23  127 | -  13 | 16  134 | -  19 | 22  163 | -  14 | 14  107 | -  14 | 18  127 |
| Beta-Proteobacteria | *Candidatus* Accumulibacter  *Comamonadaceae\**  *Nitrosomonadaceae\**  *Nitrosomonas*  *Dechloromonas*  *Zoogloea*  Unclassified | 4690  505  101  47  -  38  849 | 650  186  31  19  -  126  646 | 4750  606  108  43  29  34  800 | 792  198  28  12  20  107  596 | 4807  580  136  71  39  48  618 | 841  173  19  12  28  114  623 | 4898  741  143  76  34  38  675 | 775  215  31  16  55  127  646 | 4449  583  142  72  32  35  564 | 713  188  36  21  40  91  587 | 4719  603  126  62  34  39  701 | 754  192  29  16  36  113  620 |
| Gamma-Proteobacteria | *Acinetobacter*  *Aeromonas*  *Lysobacter*  *Rheinheimera* | 15  -  -  - | 26  85  35  - | 21  -  -  - | 36  101  46  - | 28  -  -  - | 17  82  37  - | 20  -  -  - | 29  92  66  - | 17  -  -  - | 20  92  43  - | 20  -  -  - | 26  90  45  - |
| Nitrospira | *Nitrospira* | 19 | 64 | 17 | 96 | 26 | 96 | 20 | 86 | 25 | 103 | 21 | 89 |
| Acidobacteria | *Chloracidobacterium* | - | 113 | 26 | 141 | 14 | 127 | 21 | 114 | 21 | 127 | 21 | 124 |
| Cyanobacteria\*\* | Unclassified | 19 | 33 | 24 | 48 | 13 | 44 | 23 | 52 | 27 | 75 | 21 | 50 |
| Cytophagia | *Cytophagaceae\**  Unclassified | -  - | 68  102 | -  - | 55  101 | -  - | 62  170 | -  - | 48  189 | -  - | 56  170 | -  - | 58  146 |
| Flavobacteriia | *Flavobacterium*  *Cloacibacterium* | -  - | -  - | -  - | -  - | -  - | -  - | -  - | -  - | -  - | -  - | -  - | -  - |
| Phycisphaerae | Unclassified | 21 | 79 | 18 | 116 | 26 | 118 | 24 | 88 | 28 | 113 | 23 | 103 |
| Saprospirae | *Chitinophagaceae\**  *Saprospiraceae\** | -  66 | 634  1679 | -  46 | 629  1431 | -  43 | 744  1538 | -  46 | 765  1571 | -  158 | 641  1573 | -  72 | 683  1558 |
| Unclassified | *Caldithrix* | 152 | - | 138 | 15 | 152 | 15 | 240 | 21 | 231 | 18 | 183 | 17 |

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Class | Genus | C1 | | C2 | | C3 | | Mean (C1-C3) | |
|  |  | RNA  (n=8592) | DNA  (n=9078) | RNA  (n=8584) | DNA  (n=9167) | RNA  (n=8839) | DNA  (n=9345) | RNA  (n=8714) | DNA  (n=9234) |
| Alpha-Proteobacteria | *Rhodobacteraceae\**  *Woodsholea* | 37  107 | 143  112 | 27  87 | 149  94 | 33  89 | 144  90 | 33  93 | 145  97 |
| Beta-Proteobacteria | *Candidatus* Accumulibacter  *Comamonadaceae\**  *Nitrosomonadaceae\**  *Nitrosomonas*  *Dechloromonas*  *Zoogloea*  Unclassified | 23  1250  199  59  531  246  2838 | 23  431  30  -  168  340  2459 | 37  894  160  48  361  220  3787 | 39  370  26  12  665  430  2188 | 97  735  237  86  507  206  4104 | 56  354  32  15  713  412  2458 | 64  904  208  70  477  220  3576 | 44  377  30  14  565  399  2368 |
| Gamma-Proteobacteria | *Acinetobacter*  *Aeromonas*  *Lysobacter*  *Rheinheimera* | 871  13  60  61 | 440  287  45  57 | 696  11  42  44 | 331  292  88  45 | 829  21  73  32 | 373  327  153  51 | 806  17  62  42 | 379  308  110  51 |
| Nitrospira | *Nitrospira* | 167 | 389 | 138 | 417 | 109 | 241 | 131 | 322 |
| Acidobacteria | *Chloracidobacterium* | 579 | 434 | 453 | 299 | 306 | 242 | 411 | 304 |
| Cyanobacteria\*\* | Unclassified | 32 | 21 | 36 | 13 | 24 | 13 | 29 | 15 |
| Cytophagia | *Cytophagaceae\**  Unclassified | -  61 | 44  393 | -  111 | 69  451 | -  74 | 72  434 | -  80 | 64  428 |
| Flavobacteriia | *Flavobacterium*  *Cloacibacterium* | -  20 | 105  172 | -  15 | 117  216 | -  17 | 103  188 | -  17 | 107  191 |
| Phycisphaerae | Unclassified | 53 | 64 | 37 | 60 | 47 | 48 | 46 | 55 |
| Saprospirae | *Chitinophagaceae\**  *Saprospiraceae\** | 20  - | 859  55 | 16  - | 886  41 | 23  - | 932  69 | 21  - | 902  59 |
| Unclassified | *Caldithrix* | - | - | - | - | - | - | - | - |

\* Family, \*\* Phylum, - less than 10 sequences

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