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Multiscale investigation of a symbiotic microalgal-integrated fixed film activated sludge (MAIFAS) process for nutrient removal and photooxygenation

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Abstract

The Integrated Fixed-Film Activated Sludge (IFAS) process is an advanced biological wastewater treatment process that integrates biofilm carriers within conventional activated sludge to uncouple the sludge retention time for nitrifiers and heterotrophic bacteria. In this study, we incorporated microalgae into the IFAS configuration for photo-oxygenation and evaluated the symbiotic reaction between microalgae and bacteria for both suspended solids and IFAS biofilm media. In a sequencing batch mode, the microalgae-IFAS system removed more than 99% ammonia and 51% phosphorous without the need for mechanical aeration. Biofilm microprofiles revealed localized photo-oxygenation by the algal biofilm and nitrification by nitrifiers on the IFAS media. Genetic sequencing showed that the addition of microalgae to the IFAS system promoted significant changes in the bacterial community structure and altered metabolic activity of several bacterial groups. Overall, this research represents a novel strategy for reducing energy consumption while meeting stringent effluent standards using a hybrid symbiotic microalgae-IFAS technology.

Keywords

Biofilm; Integrated fixed-film activated sludge (IFAS); Microalgae; Microelectrodes; photo-aeration

1. Introduction

As the human population increases and environmental requirements become more stringent, the need for sustainable water management treatment systems that meet regulatory standards and reduce energy consumption has become a top priority in the water industry (EPA, 2006). To address these issues, microalgal treatment systems have been studied as a low-cost, environmentally friendly, wastewater treatment alternative when compared to conventional wastewater treatment processes. Algae's nutritional and chemical requirements offer

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opportunities for advanced bioremediation and biofuel production by integrating industrial and municipal utilities with algal systems for a holistic approach to managing urban resources.

In recent years, several studies have coupled algal photosynthesis with conventional biological nutrient removal processes. In these systems, algal photosynthesis is used to dramatically decrease energy consumption by reducing energy costs associated with mechanical aeration (i.e., 45–75% of plant energy costs) (Rosso et al. 2008; González et al., 2008; Karya et al., 2013). For example, Karya et al. (2013) achieved full ammonia removal from wastewater (50 mg NH_4^+-N L^{-1}) without mechanical aeration and found that 85% of the removal was due to nitrification. Likewise, Wang et al. (2015) used a microalgalbacterial consortium to remove more than 90% total nitrogen (TN) from digested swine manure (300 mg NH_4^+-N L^{-1}) and contributed to 80% of removal to nitritation/denitritation. However, these systems rely on fast growing microalgae to supply oxygen and slow growing nitrifying bacteria for nitrification/denitrification; thereby limiting solid retention times (SRTs) that can be used to support both organisms. Ideally, the SRT for algae and nitrifying bacteria should be uncoupled to improve nutrient removal efficiencies.

Over the past two decades, many wastewater treatment plants in the United States (U.S.) have found that fixed film technologies (i.e., moving bed bioreactors [MBBR] and integrated fixed film activated sludge [IFAS]) provide an effective alternative for expanding or improving wastewater treatment, with respect to nitrification, due to its relatively small footprint (Onnis-Hayden et al. 2011). This is because the nitrifying bacteria tend to establish themselves on biofilm carriers and thus can be retained even when suspended nitrifiers would be washed out of the system (e.g., at low temperatures or short solids retention time [SRT]). While both MBBR and IFAS systems are suitable for biological N removal through nitrification, IFAS systems can be optimized for both biological N and P removal because the SRT for nitrifiers and polyphosphate-accumulating organisms (PAOs) are uncoupled. Previous research has demonstrated that nitrifiers colonized IFAS media while PAOs existed mainly in suspension (Kim et al., 2010; Sriwiriyarat and Randall, 2005; Onnis-Hayden et al., 2011). By applying this concept to an algal-bacterial wastewater process, it is hypothesized that algal biofilm can be formed on the IFAS media and provide oxygen sufficinetly to nitrifying biofilm, while maintaining a SRT suitable for P removal by suspended algal-bacterial consortium.

This study represents a novel strategy for achieving net energy production while meeting stringent effluent standards using a hybrid symbiotic microalgae-based IFAS (MAIFAS) technology. The primary objective of this study was to better understand symbiotic interactions between fixed film and suspended populations of microalgae-based biofilm processes that promote N and P removal with low energy consumption. Mechanisms of N and P removal through symbiosis of algae and bacteria without aeration were elucidated using multiscale investigations which include microelectrodes, next-generation molecular methods, models, and a series of batch studies.

2. Materials and Methods

2.1. Microalgae cultivation, bacteria inoculation, and synthetic wastewater preparation

Chlorella vulgaris (UTEX 2714, UTEX Algae Culture Collection, Austin, TX) is commonly used to study microalgal wastewater treatment (De-Bashan et al. 2002, Wang et al. 2010) and thus was selected as a model microalga in this study. The *C. vulgaris* strain was grown in 1L glass bottles (13951L, Corning Inc., Corning, NY) containing 500 mL of Bold's basal medium (BBM). The bottles were incubated at room temperature (23°C) under continuous cool-white fluorescent light illumination of 20 μmol m^{-2} S^{-1} photosynthetically active radiation (PAR) (Apollo Horticulture T5, 6400K Fluorescent bulbs) and stirred at 50 rpm. Initially, the culture was aerated using an aquarium air pump to supply CO_2 to the algae. After reaching stationary phase, the culture was used to inoculate SBR experiments. The activated sludge was collected from the return activated sludge of a local wastewater treatment plant (Iron Bridge, Orlando, FL) for seeding the SBRs. Synthetic wastewater with the following composition was used as a growth media: 240 mg/L sodium acetate, 57.5 mg/L NH_4Cl, 51 mg/L K_2HPO_4, 83 mg/L $MgSO_4$, 13 mg/L $CaCl_2$, 65 mg/L yeast extract and 65 mg/L beef extract equivalent in total to 300 mg COD/L, 30 mg TN/L and 10 mg P/L (Jabari et al. 2014). 500 mg/L $NaHCO_3$ was also added to provide sufficient alkalinity for nitrification.

2.2. Photo-SBR

Three lab-scale SBRs (4L) were constructed with plexiglass and operated in the sequence of fill, mix, aerate, idle, settle, and decant phases using a programmable logic controller (PLC) (Chrontrol, San Diego, CA) (Fig. 1 and Fig. S1). The reactors were operated at room temperature (22–24 °C) and in 12 h cycles consisting of feeding (10 min), anaerobic step (80 min), aerobic step (300 min), anoxic step (150 min), aerobic step (60 min), settling (90 min) and decanting (30 min). 40% of the bulk working volume was filled with AnoxKaldnes (K1) biofilm carriers (Veolia, Lund, Sweden) (specific surface area of 500 m^2/m^3) in two IFAS SBRs (MAIFAS and IFAS control). The third reactor with no media was used as a negative control. The reactors were operated with 12 h hydraulic residence time (HRT) and 30 d $SRT_{suspended}$ (wasting during the aerobic phase). The pH was not controlled and ranged from 7.4 to 8.1 during the 12 h SBR cycle. All three SBRs were mixed during the anaerobic, aerobic and anoxic phases using an overhead stirrer.

Before experiments, the two IFAS reactors were first inoculated with activated sludge for 60 days using the synthetic wastewater to develop a nitrifying biofilm on the K1 media. During this time, all three reactors showed 71 to >99% ammonia removal, 66 to 97% COD removal and 0 to >99% TP removal. After biofilm formation on the K1 media was observed, Phase I began with the addition of 500 mg/L *C. vulgaris* to the MAIFAS and suspended control SBRs. The SBRs seeded with algae were exposed to fluorescent light (76.2 μmol m^{-2} S^{-1} PAR) during aerobic phases. Air was supplied to all reactors during Phase I to develop the nitrifiers. A Chl. *a* to biomass ratio above 10.25 mg/g was designed to provide enough oxygen by green algae to the system for nitrification to occur (Karya et al. 2013); however, even after 75 days (Phase I), the Chl. *a* to biomass ratio was still under 6 mg/g and thus the wastewater composition was adjusted to promote microalgal growth (Phase II). Ammonia

and P concentrations were increased to 70 mg N/L and 20 mg P/L, respectively, while COD was decreased to 150 mg/L. During Phase II, air supply to the algae reactors were turned off to evaluate the capability of microalgal photo-oxygenation. The SBRs were operated under Phase II conditions for 75 days.

2.3. Analytical methods

Samples (50 mL) were taken from the effluent during the withdrawal stage twice a week for water quality analysis. The samples were filtered using 45 μm glass fiber filters (934-AH, Whatman, city and state) and analyzed for pH, ammonia, TP, and COD. pH and ammonia were measured using pH and ammonia probes (Intellical™ pH Electrode PHC20; IntelliCAL™ ammonia probe, ISENH318101, Hach, Loveland, CO). TP was measured using Hach Method 8180 (ascorbic acid method test) and COD was measured using Hach Method 8000. Generally, duplicate samples were processed during each sampling point. Additionally, hourly samples were taken through a 12hr SBR cycle and analyzed for nitrate, nitrite, and DO. Nitrate and nitrite were measured using the cadmium reduction method (Hach Methods 8039 and 8192, respectively). DO was measured using a DO probe (407510, Extech, Nashua, NH). Biomass was determined using the Standard Methods (APHA, AWWA, and WEF, 1999) for Solids (SM 2540).

Chlorophyll *a*, which was used to monitor algal growth, was measured using a modified version of the SM10200 H.2.b. method (APHA, AWWA, and WEF, 1999). Briefly, a 5 mL sample was centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded and 5 mL of 96% methanol was added to the remaining pellet. This mixture was vortexed for 10 minutes and incubated at 60 °C for 15 minutes. The sample was then cooled at 4°C for 30 minutes and centrifuged at 13,000 rpm for 10 minutes. The supernatant's absorbance at 666 and 653 nm was measured using a spectrophotometer (DR900, Hach) to determine Chl. *a* based on equation (1).

$$\text{Chlorophyll } a \text{ (mg/L)} = 15.65A_{666} - 7.34A_{653} \quad (1)$$

2.4. Microprofiling

DO concentration and pH microprofiles were measured using a DO microsensor (tip diameter: 50μm) and a pH microsensor (tip diameter: 10μm) (UNISENSE A/S, Denmark). Ammonia concentration microprofiles were measured using a fabricated ammonia ion selective microelectrode (tip diameter: 30μm) following methods described in Lewandowski (2014). The DO microsensor was calibrated in respective oxygen saturated (aeration: 8.6 mg O_2/L at 23°C) and oxygen depleted (nitrogen bubbling: 0% DO) artificial wastewater. The pH microelectrode was calibrated in standard buffer solutions (pH 4, 7, and 10, Fisher Scientific, Hampton, NH). The ammonia microsensor was calibrated in artificial wastewater with varying ammonia chloride concentrations. Microsensors were calibrated before and after each profile.

For microprofile measurements, a K1 biofilm carrier was removed from the reactor, gently cut in half using a scalpel (Disposable Scalpel No. 10, Thermo Scientific, Waltham, MA),

and placed in a customized flow cell that allowed a flow of wastewater over the biofilm at a rate of 2 mL/min (Lee et al. 2010). The sample was held in place using a clamp (VTHH, Veleman Inc., Fort Worth, TX). The positioning and movement of the microsensor tip in the sample was accomplished using a three-dimension (3D) micromanipulator (UNISENSE A/S, Denmark) and observed using a stereomicroscope with a CCD camera (World Precision Instruments, Sarasota, FL). A Ag/AgCl reference electrode (MI-401, Microelectrodes Inc.) was positioned in the flow cell using a helping hand (VTHH, Veleman Inc.) and a lab jack (Model 110, Swiss Boy lab jack, Fisher Scientific) was used to center the biofilm within the view of the stereomicroscope. The microprofile measurements were conducted in a Faraday cage (81-334-04, Technical Manufacturing Co., Peabody, MA) to minimize electrical interference. A florescent light was used to provide 76.2 μmol m^{-2} S^{-1} of light (the same as the MAIFAS SBR operation) to the microalgae integrated fixed biofilm carrier during profiling measurements. A photo of the experimental set up is shown in Figure S2. Microprofile measurements were performed from 3,000μm above to the surface of the K1 media to the surface.at every 100 μm with 5 seconds intervals between each measurement. Two replicate profiles were taken for each parameter.

2.5. Analysis of bacterial community

2.5.1 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) biofilm samples as previously described (Pitkaänen 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. 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) as 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 (Caporaso et al. 2011). Sequencing was performed at the Cincinnati Children's Hospital Medical DNA Sequencing and Genotyping Core facility.

2.5.2. 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. This includes reads that did not form contigs, deviated considerably from the expected PCR size product, had ambiguous bases, or 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. 2012) 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.

3. Results

3.1. Nutrient removal and biomass growth

3.1.1. Phase I. Mechanical aeration—During Phase I, all reactors were mechanically aerated during aerobic phases. Fig. 1 shows influent and effluent pH, ammonia, COD and TP changes during the experiment. After the inoculation of microalgae, it took 37 days for the system to show consistent nutrient removal. Before this, the IFAS control exhibited excellent COD, ammonia, and TP removal (95%, 80%, and 89%, respectively) but reactors containing microalgae (MAIFAS and suspended) displayed less than 60% removal for COD, ammonia, and TP. However, once the algae was acclimated, no detectable ammonia and no more than 26 mg L−1 COD were found in the effluent of all three reactors. Likewise, TP removal was above 70% for all reactors for the end of Phase I with the exception of a one-week period where TP exceeded 5 mg P L−1 in the effluent of the suspended reactor. The effluent pHs of the SBRs containing microalgae were found to be higher than the IFAS control, particularly in towards the end of Phase I. For example, the effluent pH of the MAIFAS and suspended reactors were approximately 8.2 for days 50–75; however, the IFAS control and influent was about 7.6. This could be due to the algae consuming CO2, which would increase the pH. Fig. 2 shows the characteristics of the suspended biomass in each reactor over the 150-day experiment. During Phase I, all SBRs exhibited decreasing mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) for the first 30 days after inoculation of C. vulgaris. The suspended SBR had a starting MLSS of 5250 mg L−1 and decreased to 3500 mg L−1, the MAIFAS SBR had a starting MLSS of 2000 mg L−1 and decreased to 1000 mg L−1, and the IFAS control reactor has a starting MLSS of 2000 mg L−1 and decreased to 1800 mg L−1 after the first 30 days. After this initial decrease, the MLSS concentration was consistent for the remainder of Phase I. The MAIFAS and IFAS SBRs had significantly lower suspended biomass compared to the suspended SBR; however, biofilm biomass can be estimated to contribute approximately 4000 mg L−1 additional biomass to the MAIFAS and IFAS reactors (considering a biofilm density of 20 mg cm−3 (Ro and Neethling, 1991)); therefore, the total biomass concentrations in all three reactors were estimated to be 3500 mg L−1, 4000 mg L−1, and 4800 mg L-1 for the suspended, MAIFAS and SBRs, respectively. While all reactors displayed excellent nutrient removal throughout Phase I, the reactors were all supplied with oxygen during the aeration phases of the SBR sequence. This may have impeded the growth of microalgae. During Phase I, the growth of microalgae was limited to 5.76 mg g−1 in the MAIFAS reactor and 1.76 mg g−1 in the suspended reactor (Fig. 2(d)).

3.1.2. Phase II. Photo-aeration—In Phase I, it was found that microalgal growth may be hindered by competition for nutrients with the activated sludge. Therefore, in Phase II,

nutrient concentrations were increased to 70 mg NH3-N L−1 ammonia and 20 mg P L−1 total phosphorus (TP) in an attempt to further support microalgal growth. Furthermore, preliminary batch experiments demonstrated that growth rates slow down significantly when C. vulgaris opts for heterotrophic growth and results in poor photo-aeration. Therefore, influent COD was reduced to 150 mg L−1 and the MAIFAS and suspended reactors were no longer mechanically aerated during Phase II.

The change in experimental conditions (i.e., increase in influent N and P concentrations, reduction of COD input, and cessation of mechanical aeration) clearly increased microalgal growth. Chl. α concentration increased from 3.28 to 44.95 mg/L and 4.15 to 9.53 mg/L in the suspended and MAIFAS SBRs, respectively. This growth in algae also increased the Chl. α to biomass ratio to 20.3 and 13.01 mg/g in the MAIFAS and suspended reactors, respectively. MLVSS decreased by 43% in the IFAS control and 26% in the MAIFAS. This decrease was likely attributed to the decreased influent COD concentration in Phase II. While there was an initial decline in nutrient removal during Phase II in the MAIFAS and suspended SBRs, nutrient removal was relatively improved after 40 days in Phase II. The control IFAS reactor was still able to remove all 70 mg N-NH_3/L ammonia of influent. The adjusted experimental conditions impeded P removal in the control IFAS reactor due to the decreased suspended biomass (MLVSS) (< 1,000 mg/L) (Fig. 2(a)); however, the reactors with algae, MAIFAS and suspended, demonstrated 51 and 98% P removal, respectively. Furthermore, the algae bacteria consortia showed excellent settling with a sludge volume index (SVI) ranging from 74 to 160 with less than 1 mg/L Chl. α in the effluent.

There was a slight increase in effluent pH in the suspended reactors from Phase I (pH 8.2) to Phase II (pH 8.5) (Fig. 1(a)), indicating algal growth and increased photosynthetic activity in the reactors. While pH increases may have stripped some ammonia from the system, the highest pH observed during the aerated phase was 8.5, indicating that the majority of the ammonia removal was biological N removal, not ammonia stripping.

3.2. Photo-aeration using microalgae

DO was carefully monitored throughout the experiments (Phase I and II) to evaluate microalgae photo-aeration as an alternative to energy-intensive mechanical aeration. Bulk DO concentration was monitored without mechanical aeration in the algae reactors during the entire SBR cycle for each reactor at 30 d, 60 d and 90 d after inoculation (Fig. 3). The IFAS control reactor was mechanically aerated during the aerobic phases of the SBR cycle; hence, the DO reached saturation during this phase. This high concentration of DO is not typical of real WWTPs; but was observed in this study due to the complete consumption of biochemical oxygen demand (BOD) before the end of the aeration phase. For the reactors supported by photo-oxygenation, it appears that the microalgae do not provide enough DO for nitrification at 30 and 60 d (Phase I), requiring the change of influent water quality and more time for stable algal growth in the system. At 90 d (Phase II), DO concentration increased to 1.3 mg O2 L−1 and 0.6 mg O2 L−1 in the MAIFAS and suspended SBRs, respectively, indicating photo-aeration. This finding demonstrates that microalgae can provide sufficient oxygen required for nitrification and COD removal particularly when the Chl. a to biomass ratio exceeds 7.9 mg g−1 (MAIFAS Chl. a to biomass ratio after 90 days).

A study by Wang et al. (2015) found similar results for a photo-SBR that was used to process the liquid fraction of anaerobically digested swine manure. They found that a Chl. a to biomass ratio of approximately 24 mg g−1 was able to supply 74% of the required DO for nitrification.

While DO concentrations in the bulk provided some indication of photo-aeration, direct oxygen transfer measurements within a microalgal-bacteria biofilm provides valuable mass transport information. Therefore, DO microsensors were used to measure DO concentration microprofiles from the bulk into the biofilms of the MAIFAS and IFAS SBRs which were taken after 40, 80, 130, and 150 d of operation (Fig. 4). The microprofile measurements were conducted between dark and light conditions to confirm the photo-aeration by algal biofilm. Biofilm thickness was 600, 1300, 1800, and 2100 μm at 40, 80, 130, and 150 days of operations, respectively. DO concentration microprofiles after 40 days of biofilm growth (Fig. 5(a)) showed a decrease in DO from 3.0 mg O2 L−1 in the bulk phase to 0.2 mg O2 L −1 at the substrate with a 100 μm diffusion boundary layer (DBL) which indicates the activity of aerobic bacteria (nitrifiers and heterotrophs) within the biofilm. When the light was turned off, there was an initial decrease by 0.5 O2 mg L−1 from 1500 to 600 μm, after which the O2 concentration decreased to 0.32 mg O2 L−1. After 80 d, biofilm thickness was increased to 1300 μm. The DO profile exhibited a 1 mg O2 L−1 increase in DO at the biofilm surface (Fig. 4(b)) by microalgal photosynthetic oxygen production. After 130 d of operation (55 days of Phase II), DO concentrations increased to 6.8 mg O2 L−1 at the surface of the biofilm with the lights on. ΔDO between light on and off was 3.3 mg O2 L−1 (Fig. 4(c)). This large amount of oxygen production at the biofilm surface made it possible for DO to penetrate into the whole biofilm where DO was present at the substratum (0.3 mg O2 L−1) which means the underlying nitrifying biofilm had ample supply of oxygen for nitrification. Without light, the bulk DO penetrated only 69% of the total biofilm thickness. This outcome shows that there was an algal biofilm present on top of the bacterial biofilm which can assist in photo-aeration and this localized algal photo-aeration improved the utilization of aerobic bacteria throughout the biofilm depth, which was not possible through mechanical aeration. Altogether, these findings provide evidence of the feasibility of a microalgae-based IFAS wastewater treatment process to achieve low energy consumption while meeting stringent effluent standards using symbiotic microalgae fluidized media. A microscopic cross-sectional image of the biofilm showed the bottom layer was comprised mostly of a dense mixed culture nitrifying biofilm where the top layer was a mixture of algae and heterotrophic bacteria. In addition, DO consumption within the biofilm was faster at the base of the biofilm (0–600 μm) compared to the top 600 μm of the biofilm (Fig. 4). This clearly shows well-defined stratification of microbiological communities between the top and bottom layer. This configuration of bacterial communities seems possible considering nitrifying bacteria are known to be photosensitive (Kaplan et al., 2000).

After 150 d, the DO concentration microprofiles of MAIFAS biofilms displayed the loss of a photo-oxygenating layer on the surface of the biofilm, showing no difference between light and dark conditions with a similar DO decreases from 3 mg O2 L−1 in the bulk to 0 mg O2 L−1 at 800 μm above the substrate surface (Fig. 4(d)). Therefore, the lower nitrifying biofilm appears to be unable to get oxygen for nitrification. This is consistent with the fact that ammonia removal decreased from 99% on day 130 to 11% on day 150 (Fig. 1(b)). It is

still unclear why there was a significant loss of the phototrophic biofilm at this time of operation. A possible explanation for the loss of the photo-oxygenating microalgla biofilm would be algal biofilm sloughing due to the precedent nutrient starvation (100% ammonia removal) (Schnurr et al., 2013), decreasing algal growth from days 129 to 141 (Fig. 2(c)).

3.3. Ammonia removal mechanism in algal and bacterial biofilms

To further understand the interactions between algal and bacteria biofilms, pH and ammonia concentration microprofiles were measured in Phase II of MAIFAS operation. pH deceases from 7.3 to 6.7 in the lower 1,000 μm of the biofilm when the light was on (Fig. 5(a)), indicating consumption of alkalinity and nitrification. In addition, there was a slight pH increase (600 μm above the biofilm surface), from 7.3 to 7.4, which indicates photosynthesis. Likewise, ammonia concentration decreased from 72 mg N/L to 67 mg N/L in the lower 250 μm of the biofilm (Fig. 5(b)). This finding was interesting because there was no consumption of ammonia in the upper portion of the biofilm meaning the algal portion of the biofilm was not consuming ammonia. Hence, the role of algae biofilm in the MAIFAS reactor is mostly aiding photo-oxygenation.

3.4. Genetic diversity of algae-bacteria consortia for wastewater treatment

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 between the MAIFAS and the IFAS reactors. The sequencing data suggested that there were some similarities in bacterial composition between the reactors. Each reactor included members of bacterial phyla such as Proteobacteria (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 types. For example, while members of the beta-proteobacteria were among the most abundant groups in both reactors, some of the species were more prevalent in one reactor type. Specifically, *Candidatus* Accumulibacter was predominant in the MAIFAS reactor but barely detected in the IFAS reactor. In contrast, *Dechloromonas* represented >5% of the sequences in the IFAS reactor versus < 0.4% in the MAIFAS 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 twice 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 while 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 the role of *C.*

abyssi in the MAIFAS reactor is unknown at this point, genome sequencing analysis of has revealed 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 (Pitkaänen 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, Kapoor et al. 2015b). 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 while over six times more abundant in the rRNA libraries than in the rDNA libraries in the MAIFAS reactor, suggesting that this bacterial group may be playing an active role in P removal in the algae-seeded reactors (MAIFAS). Previous studies have reported *Candidatus* Accumulibacter *phosphatis* as a dominant member of enhanced biological phosphorus removal (EBPR) sludge microbial communities (Flowers et al. 2013). In fact, *Candidatus* Accumulibacter is capable of P removal in wastewater enrichments employing different oxygenic conditions (Camejo et al. 2016). Also related to P removal were the dynamics of *Dechloromonas spp*. whose relative abundance significantly decreased in the MAIFAS samples. Members of this genus have been shown to accumulate polyphosphate. As other potential PAO were not detected or were present in very low numbers, the results of this study implicate *Candidatus* Accumulibacter and *Dechloromonas* as the primary P removing bacteria in the MAIFAS and IFAS, respectively. The role of difficult to classify beta-proteobacteria cannot be discarded. 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 *Candidatus* Accumulibacter, resulting in an increase of P removal.

3.5. Micro-algae bacteria consortium for nutrient removal in synthetic wastewater

One of the benefits of an IFAS process is the ability to reduce the footprint compared to a traditional activated sludge process. Considering an equivalent reactor size, the MAIFAS system removed more than 99% ammonia and 51% P without the need for mechanical aeration, a marked improvement over the suspended microalgae sludge control, which only removed 57% ammonia and 49% P from synthetic wastewater.

Ammonia removal in MAIFAS and suspended microalgae-bacteria reactors were comparable to other microalgae wastewater treatment studies. He et al. (2013) observed 97% ammonia removal in municipal wastewater (29–174 mg NH_4^+-N/L) and Zhao et al. (2014) reported 90% TN removal in landfill leachate (261 mg NH_4^+-N/L) using a microalgae-bacteria consortium. N removal in these systems was achieved through N assimilation by biomass and nitrification/denitrification pathways. Su et al. (2012) also found that 61–93% of N removal was due to N assimilation. Stripping ammonia through elevated pH is another common mechanism for TN removal in microalgae wastewater treatment processes; however, the pH of the MAIFAS system in this study never exceeded 8.3, indicating that TN was mostly biologically removed. A shortcut N removal mechanism was proposed by Wang et al. (2015) where low DO and carbon concentrations favored ammonia oxidizing bacteria (AOB) over nitrite oxidizing bacteria (NOB) activity in the presence of algae (Wang et al. 2015). This removal process requires less DO and organic carbon compared to conventional biological nutrient removal (BNR) processes. While a nitrogen balance was not performed in this study, 16G rRNA gene sequencing analysis of the MAIFAS biofilms suggests that this shortcut N removal s significant in our system with relatively high abundance of AOB (1.5% *Nitrosomonadaceae*) compared to NOB (0.2% *Nitrospira*).

Biological P removal using algae-based biological processes is less common than N removal, but can be achieved. For example, Gonzales et al. (2008) found 80% P removal from swine manure wastewater using *Chlorella sorokiniana* and Posadas et al. (2013) reported 85% removal from domestic wastewater using a mixed algae culture. A biofilm study showed only a 34% removal from dairy manure using an algal turf scrubber.

In the current study, it was found that P removal was similar between the MAIFAS and suspend algae reactors in Phase II with an average of 51% removal from both reactors. Based on this observation it is likely the suspended portion of the biomass that is responsible for P removal. In fact, we found that P removal is not necessarily dependent on Chl. a to biomass ratio in the MAIFAS reactor system, suggesting that P removal may not be attributed to algal assimilation. 16 s rRNA sequencing revealed high concentrations of Candidatus Accumulibacter in MAIFAS biofilms compared to the IFAS control (55% vs. less than 1%) likely due to EBPR's contribution in P removal in microalgae systems.

3.6. Effect of Chl. A to biomass ratio on nutrient removal

Several studies have noted the importance of algae to biomass ratio for nitrification in algal-bacterial consortia (Su et al., 2012). A study by Medina and Neis (2007) demonstrated that increasing Chl. a to biomass ratios (1–34 mg chl. a/g SS) increased TN removal. In this study, ammonia removal was compared to Chl. a to biomass ratio in Phase I and II between the MAIFAS and suspended reactors (Fig. 6) to elucidate N and P removal mechanisms by algal-bacterial symbiosis. Chl. a to biomass ratio appeared to have no effect on ammonia removal in Phase I when the reactor was being mechanically aerated. This means that the activated sludge was responsible for majority of the ammonia removal under mechanically aerated conditions. During Phase II, the Chl. a to biomass ratio had impacts on ammonia removal in the MAIFAS and suspended reactors (Fig. 6(b)). Both reactors show increasing ammonia removal with increasing Chl. a to biomass ratio; however, the MAIFAS reactor had

better ammonia removal compared to the suspended reactor (Fig. 6(b)). This could be due to the fact that ammonia oxidizing bacteria (AOB) are photosensitive (Kaplan et al., 2000) and light intensity was increased for Phase II. For Chl. a to biomass ratio, both reactors followed a similar trend where for every 1 mg Chl. a g−1 VSS increase there was a 3.1% increase in ammonia removal. Therefore, microalgae are presumably responsible for removing ammonia either though assimilation or by providing oxygen for nitrification. A similar study using Scenedesumus sp. algae and bacterial flocs for wastewater treatment estimated 13% of N was removed via algal assimilation and 85% of N was removed via photo-aeration for nitrification (Karya et al., 2013).

While N removal by algal-bacterial consortia has been well studied, P removal using algal bacteria consortia is still in early stages of investigation and the mechanism for P removal is not well understood. Our data shows that when the algal-bacterial consortium was mechanically aerated (Phase I), P removal was not related to Chl. a to biomass ratio (Fig. 6(c)). This finding suggests that with mechanical aeration (Phase I), algae was not responsible for significant amounts of P removal. During Phase II, the role of algae in P removal appears to be different between the MAIFAS and suspended reactors. In the MAIFAS reactor, P removal was comparable between 35% and 51% despite Chl. a to biomass ratio (Fig. 6(d)). However, the suspended reactor showed decreased P removal with increased Chl. a to biomass ratio, indicating that unlike ammonia removal, P removal depends primarily on wasting of suspended bacteria. From the bacterial community analysis (See Section 3.4), a large amount of Candidatus Accumulibacter was found in the biofilms of the MAIFAS reactor (55% of 16S rDNA sequences). Given that P removal only occurs through wasting, it is likely that a significant Candidatus Accumulibacter population in the suspended biomass of the MAIFAS reactor was contributing to P removal.

3.7. Interactions between algal and bacteria biofilms

There is limited information on the interactions between algal and nitrifying biofilms; however, there are many studies that evaluate algal biofilms for nutrient removal. For example, algal turf scrubbers have been shown to remove an average of 1,110 mg Nm−2 d−1 (Craggs et al., 1996) and RABR have achieved nitrogen removal rates of 14,100 mg Nm−2 d −1 (Christenson and Sims, 2012). Therefore, it was expected that the algal biofilms in the MAIFAS reactor would contribute significantly to ammonia removal; however, microprofiles revealed no ammonia consumption in the algal portion of the biofilm. While the reason for this is unclear, the role of microalgae in the MAIFAS system was to provide oxygen to the nitrifying biofilm and protect the nitrifying biofilm from sloughing (Babu, 2011).

3.8. Challenges and future prospects

Integrating microalgae with IFAS processes can provide many benefits including reduced the costs of mechanical aeration, uncoupling SRTs of suspended and biofilms for reduced footprint, and potential application as feedstock for biofuel production. This study has demonstrated greater than 99% ammonia removal and 51% P removal without the need for mechanical aeration or carbon addition. However, microalgae-based wastewater treatment technologies come with inherent challenges (e.g., the need for sustained sunlight for

photoaeration must be satisfied). While the developed MAIFAS system demonstrated promising results, controlling the growth of algae can be challenging and the effect of co-growth of bacteria and algae on nutrient removal can be compounded in the operation of algal-biofilm processes. For example, when algal growth rate decreases, there was a loss of nutrient removal due to the reduced photo-aeration as well as the formation of new biofilms on top of the algal biofilms, reducing available light for photosynthesis. Alternatively, rapid algal growth can limit the growth of nitrifying and heterotrophic bacteria. It was found that this competition between algae and bacteria can lead to extended stabilization time in the MAIFAS system and significant variability in nutrient removal in the MAIFAS operation (Fig. 1). Therefore, it is important to fully understand the ecology of algal-bacterial interactions in algae-based technologies. Another potential limitation with the MAIFAS process presented in this study is the limited photoaeration using a synthetic wastewater with significant amounts of biodegradable organics. We found that C. vulgaris preferred to grow heterotrophically when presented with a COD of 300 mg/L. Therefore, this technology may be more appropriate for treatment of industrial or agricultural wastewaters with large ammonia and low organic carbon concentrations. Future work in this direction would aim to reduce the SRT to avoid the growth of heterotrophic bacteria over the fast-growing microalgae in suspended portions or select local algal strains that are better suited for maintaining healthy microbial communities. In addition, although *C. vulgaris* was used as the seed algae, it may not be the dominate algae in a mature system. Thus, time-course analysis of the algal community is required to evaluate the stability of the MAIFAS system. Lastly, this study evaluated the mechanisms of nutrient removal by an algal/bacteria consortia under a fixed source of light. Alternately, sunlight could be used to support photoaeration. Therefore, future experiments, evaluating the photo-oxygenation and nutrient removal under direct sunlight are suggested.

4. Conclusions

The removal of N and P from synthetic wastewater was investigated in a novel MAIFAS SBR over 150 days. Microalgae photosynthesis was able to provide sufficient oxygen for advanced wastewater treatment (greater than 99% ammonia and 51% P removal in the MAIFAS reactor).

A microelectrode investigation revealed localized aeration in the MAIFAS biofilms. The addition of microalgae to the IFAS system promoted significant changes in the bacterial community structure and the metabolic activity of several bacterial groups. Overall, this research represents a novel strategy for reducing energy costs while meeting stringent effluent standards using a hybrid symbiotic microalgae-based IFAS technology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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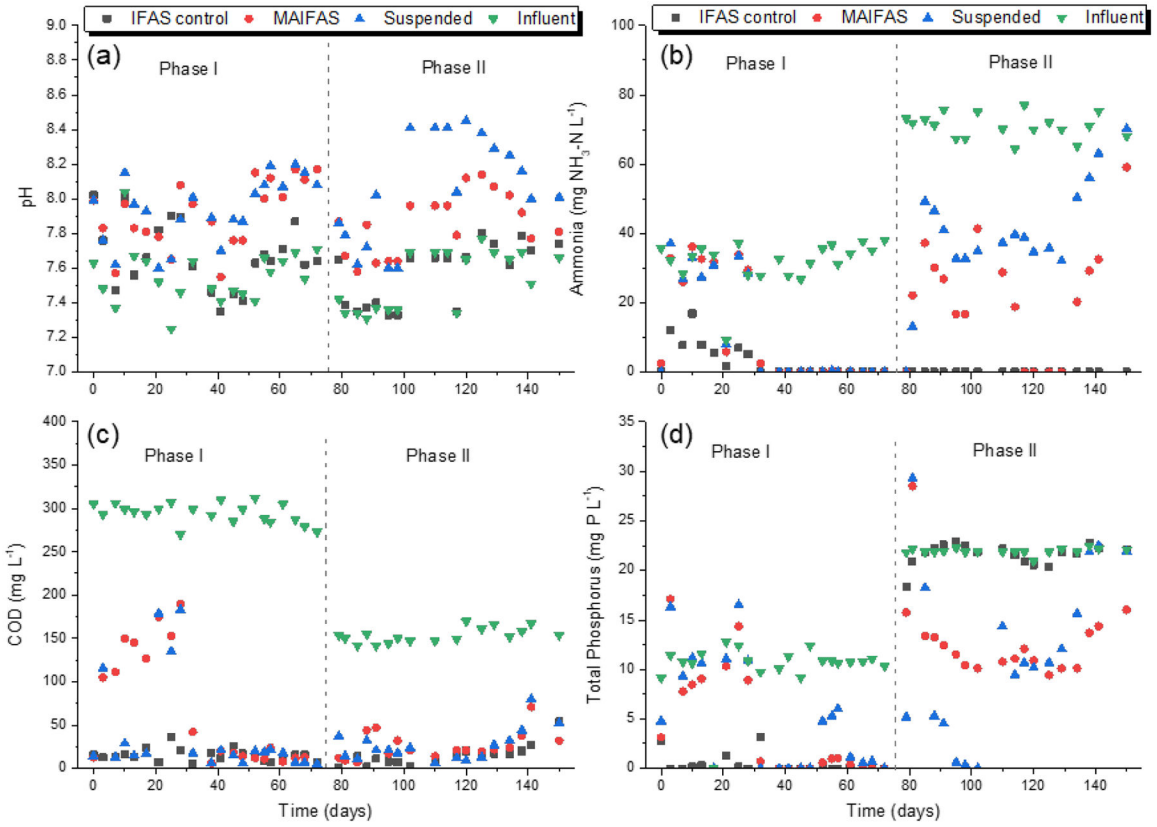

Figure 1.

(a) pH, (b) ammonia, (c) COD and (d) total phosphorus (TP) effluent changes in IFAS control, MAIFAS and suspended algae control SBRs over 150 days. Mechanical aeration was applied to all reactors during the aerobic sequence of Phase I. Only light was applied to MAIFAS and suspended reactors during the aerobic sequence of Phase II.

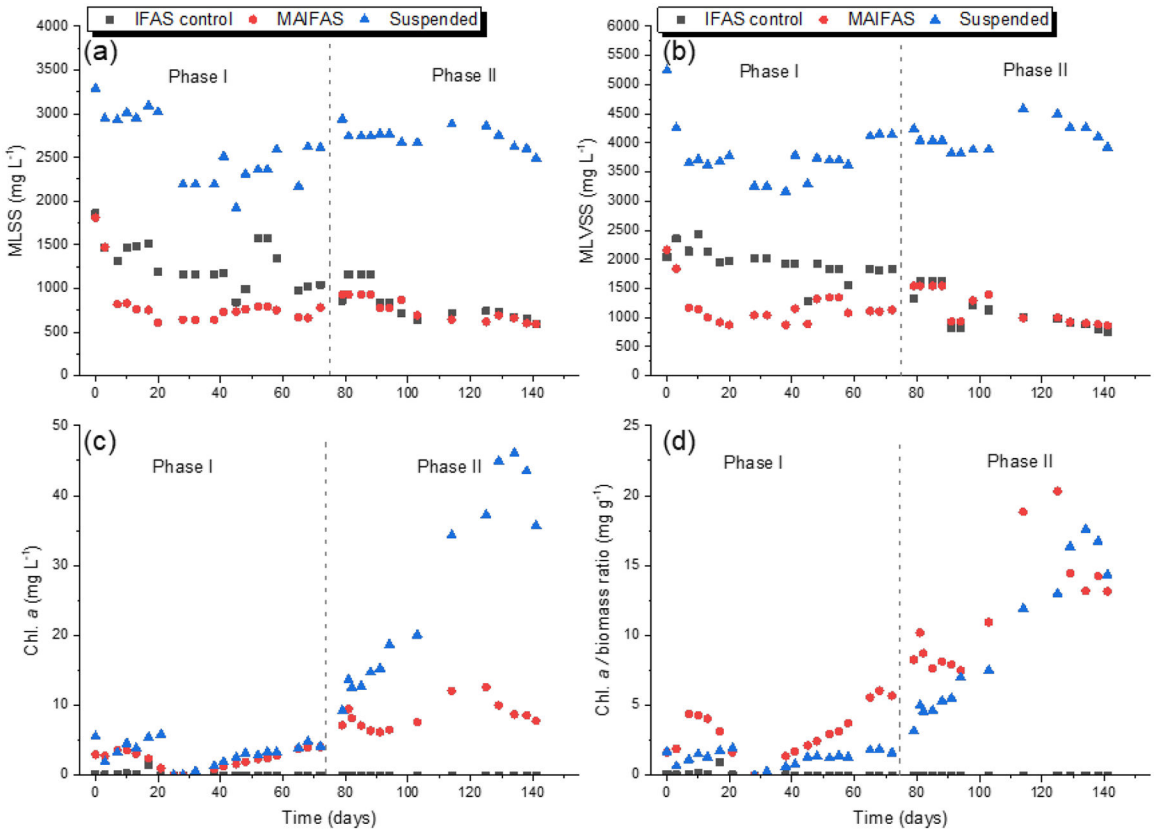

Figure 2.

(a) MLSS, (b) MLVSS, (c) Chl. *a* and (d) Chl. *a*/biomass ratio in IFAS control, MAIFAS and suspended algae control SBRs over 150 days. Biomass samples were taken during the aerobic phase. Mechanical aeration was applied to all reactors during the aerobic sequence of Phase I. Only light was applied to MAIFAS and suspended reactors during the aerobic sequence of Phase II.

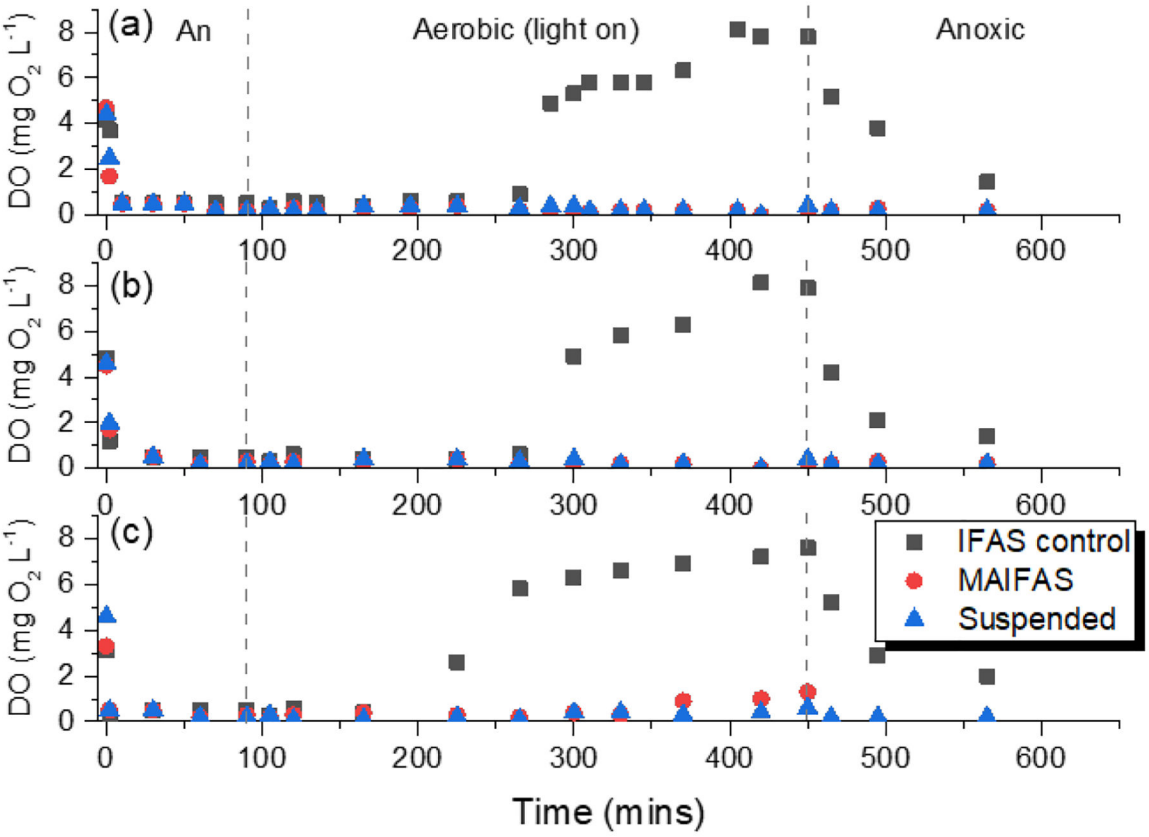

Figure 3.

DO concentrations during an SBR cycle after (a) 30, (b) 60 and (c) 90 days of growth. Mechanical aeration was applied only to IFAS control during aerobic phase. 76.2 μmol m^{-2} S^{-1} of fluorescent light (no mechanical aeration) was applied to the MAIFAS and suspended reactors during aerobic phase.

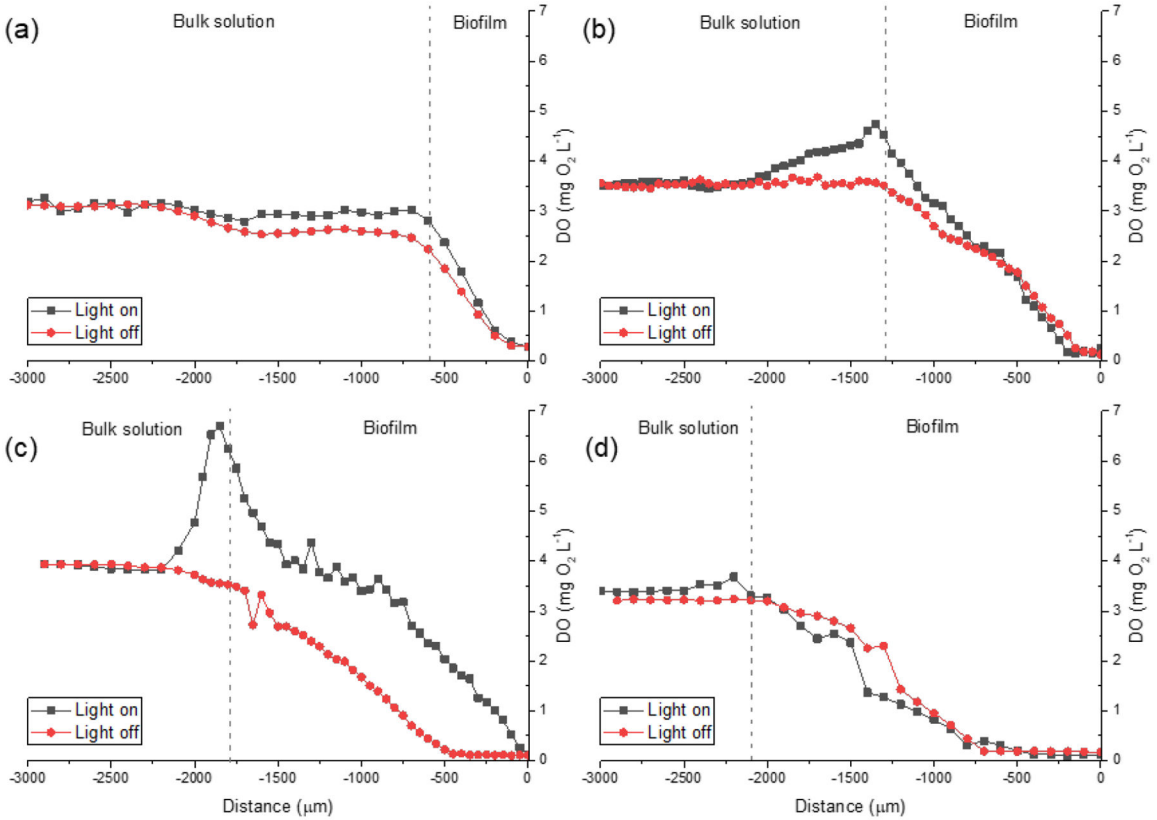

Figure 4.

DO concentration microprofiles of MAIFAS biofilms under 76.2 μmol m^{-2} S^{-1} of white fluorescent light and darkness after (a) 40 days, (b) 80 days, (c) 130 days and (d) 150 days of growth. Reference lines represent biofilm thickness over time.

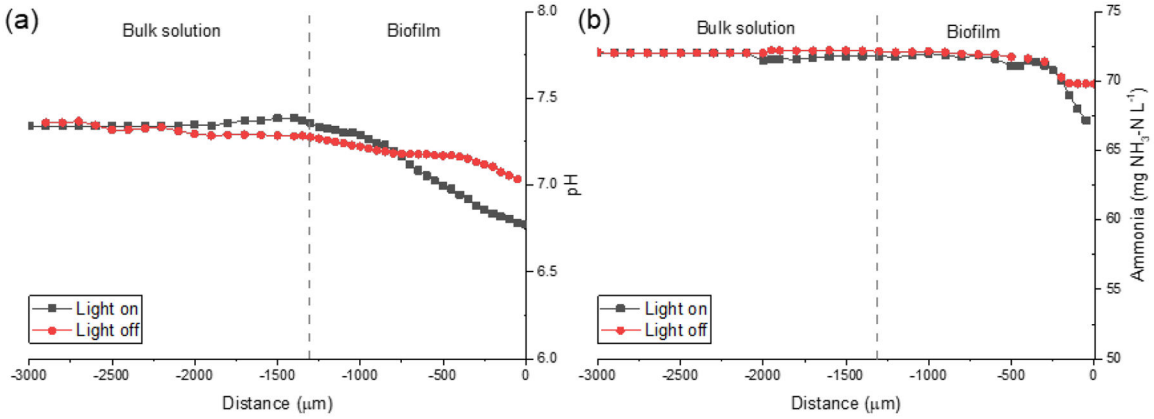

Figure 5.

(a) pH and (b) ammonia microprofiles of MAIFAS biofilms under 76.2 μmol m^{-2} S^{-1} of white fluorescent light and darkness after 140 days of growth. Reference lines represent biofilm thickness over time.

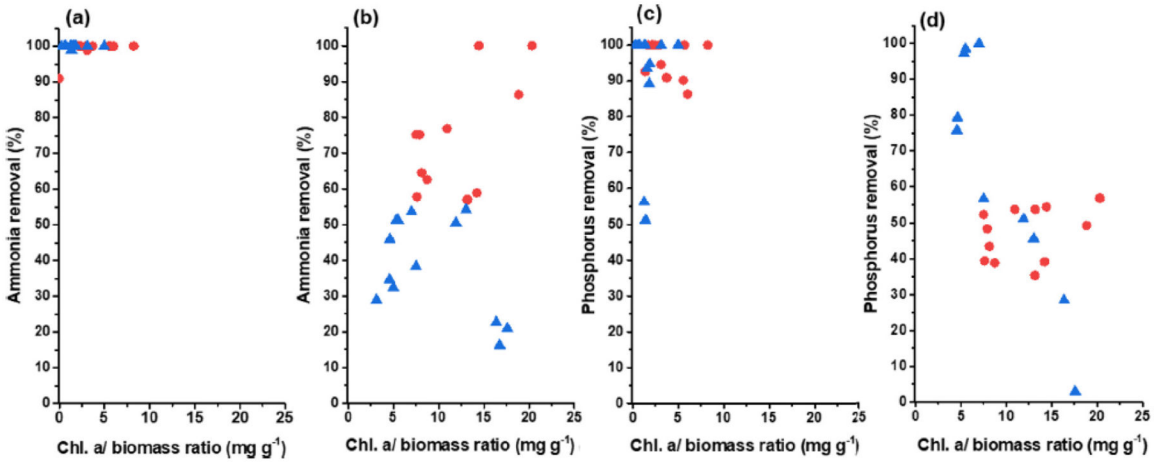

Figure 6.

Effect of Chl. a to biomass ratio on ammonia removal during (a) mechanical aeration (Phase I) and (b) photoaeration (Phase II) and P removal during (c) mechanical aeration (Phase I) and (d) photoaeration (Phase II).

Table 1.

Distribution of bacterial 16S rRNA and 16S rDNA between MAIFAS and IFAS.

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

* Family,

** Phylum, - less than 10 sequences