MICROBIAL BIOMASS GROWN ON PRIMARY TREATED WASTEWATER

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ABSTRACT

This preliminary research examined microbial biomass growth in sequence batch reactors fed primary treated wastewater from Christchurch Wastewater Treatment Plant (CWTP), New Zealand. Reactors were inoculated with indigenous microalgae (and bacteria) from oxidation ponds at CWTP. Microalgal-bacterial flocs were developed by systematically discarding the non-settleable material (supernatant) and retaining settleable solids within the reactors. Treated ammonia concentrations averaged 5.5 mg/L (as N) with a 9-day hydraulic retention time, representing a reduction of feed water concentrations by 46-100%. This ability is of significance to municipal wastewater treatment plants who struggle to remove ammonia. Activated sludge was then added to improve biomass settleability, but resulting anaerobic conditions caused a loss of aerobic bacterial activity. Bacterial community analysis using 16S ribosomal RNA gene sequencing confirmed the dominance of anaerobic genera within the settleable biomass. Highly reducing conditions within the reactors inhibited nitrification, the pH (i.e., typically < 8.0) prevented N_2 volatilisation, and some ammonification occurred, so ammonia levels decreased slowly. Activated sludge addition also caused a decrease in microalgae growth and diversity possibly due to ammonia toxicity, higher organic loading, and/or shading by excess bacteria hindering photosynthesis. Despite these effects, settleability improved with activated sludge addition. Bioflocculation and incorporation of microalgae into the activated sludge flocs were the primary mechanisms affecting biomass settleability.

KEYWORDS

Wastewater treatment, ammonia, microalgal-bacterial biomass, activated sludge, bioflocculation

1 INTRODUCTION

Much debate currently encircles the use of land-based crop biofuels, which can have negative ecological and social impacts in their production. However, biofuels derived from microalgae grown on wastewater are optimal as they can be produced sustainably and do not displace food supplies. Therefore, using microalgae as a biomass feedstock to produce biofuel may provide an effective solution to the global energy challenge while also paving the way for truly sustainable economic development within ecosystem boundaries.

Municipal wastewater contains an abundant supply of the nutrients (C, N, P) required for microalgal growth, and so, upon exposure to light, biomass production and concurrent wastewater treatment is readily facilitated (Eisenberg 1981; Banat et al. 1990; Gutzeit et al. 2005; Sreesai and Pakpain 2007). Although this field has been researched since the 1950s (e.g., Burlew 1953; Oswald and Golueke 1960), site-specific studies are necessary to thoroughly assess biomass growth before implementing full-scale biotechnologies due to the inherent variability and diversity of living systems and the differing climatic conditions under which they are studied. Moreover, significant physiological differences between microalgae strains of the same species may occur due to environmental adaptation, which can have substantial impacts on productivity and harvestability (Sheehan et al. 1998). This preliminary research investigated the growth of microalgal-bacterial biomass on primary treated wastewater from Christchurch Wastewater Treatment Plant (CWTP), New Zealand as a potential waste source of biomass feedstock for biofuel production.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SETUP

Two replicate sequencing batch reactors (SBRs) were inoculated with primary treated wastewater (2.8 L to supply nutrients and indigenous bacteria) and oxidation pond wastewater (approximately 18.4 L from Pond 6 to supply indigenous microalgae and bacteria) from CWTP. Each SBR had a 0.3-m internal diameter and a 0.4-m height and was operated in batch-fed mode with an effective volume of 21 L and a 9-day hydraulic retention time (HRT). Table 1 summarises the water quality of oxidation pond 6 and primary treated wastewaters.

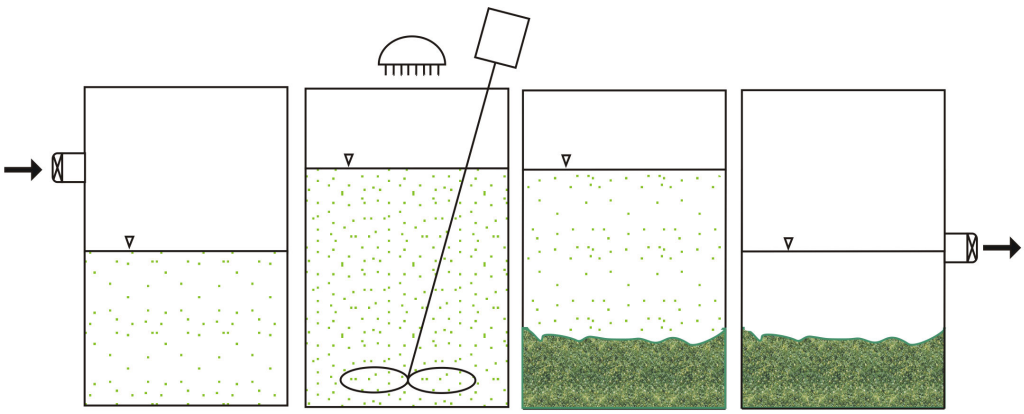
Table 1. Wastewater Characteristics[(a)]

Parameter	Unit	Oxidation Pond 6 Inoculum	Primary Treated Wastewater	Return Activated Sludge[(b)]
		Value	Mean ± s.d.	Value
Total COD	mg/L	98	479 ± 150	–
Dissolved COD	mg/L	42	202 ± 44.5	–
Total Nitrogen	mg/L	30.5	64.4 ± 14.2	–
Total Kjeldahl Nitrogen	mg/L	30.0	66.1 ± 13.4	–
Ammonia Nitrogen	mg/L	26.0	44.0 ± 7.3	–
Nitrite Nitrogen	mg/L	0.3	0.3 ± 0.0	–
Nitrate Nitrogen	mg/L	0.2	0.1 ± 0.2	–
pH	S.U.	8.2	7.5 ± 0.2	–
Total Phosphorus	mg/L	9.1	10.5 ± 1.0	–
Dissolved Reactive Phosphorus	mg/L	7.8	6.8 ± 1.1	–
Total Suspended Solids	mg/L	39	165 ± 66.0	3,900
Volatile Suspended Solids	mg/L	38	170	3,400

(a) Data provided by CWTP (2008). (b) Concentrated approximately 4-fold prior to inoculation.

Microbial (i.e., microalgal and bacterial) biomass was accumulated by draining off the non-settling material within the supernatant after each reaction phase (RP) and retaining the settled material for subsequent RPs as outlined in Figure 1. Each SBR was illuminated for 12 hr/day (i.e., 7:00 am to 7:00 pm) with a Philips 400-W incandescent bulb resulting in average photosynthetically active radiation (PAR) of 516 μmol photons $m^{-2}s^{-1}$ at the water surface, which is comparable to autumn/winter radiation in Christchurch. The cultures were maintained at a temperature of 25 ± 3 ºC using air fans. Fresh primary treated wastewater was collected every two weeks and stored at 4 ºC until required for each new RP.

Figure 1. Operating Sequence for Batch-fed SBRs



2.2 ANALYTICAL METHODS

The pH, temperature, PAR, and dissolved oxygen (DO) for each SBR were logged continuously at 15-min intervals throughout the study. The wastewater mixture was sampled at the start and end of each 3-day RP, and the discarded supernatant was also sampled at the end of each 3-day RP. Colorimetric measurements of total chemical oxygen demand (COD), ammonia (NH_3), and nitrate (NO_3) were made in accordance with Hach (2003) using a Hach DR/2000 or Hach Odyssey spectrophotometer. Total nitrogen (following digestion using a Hach DRB200) and nitrite (NO_2) were measured periodically to ascertain the nitrogen budget.

Following return activated sludge (RAS) addition, several modifications to the water quality measurements were made. Samples for total nitrogen, ammonia, nitrate, and nitrite analysis required filtering due to colour interference in RAS samples with the spectrophotometer. Since these nitrogenous constituents are inherently soluble, however, their filtered concentrations were deemed comparable to those of unfiltered samples. A Sonics Vibra-Cell cell disrupter was used to homogenise total COD samples at 30-40% amplitude for 30-60 seconds to ensure sample representativeness.

Turbidity was measured using a Hach 2100N or 2100P turbidimeter. Sludge volume index (SVI), total suspended solids (TSS), and volatile suspended solids (VSS) were analysed using conventional methods (American Public Health Association 2005). Chlorophyll (Chl) *a* was analysed according to Biggs and Kilroy (2000) and measured by accredited procedures in Cawthron and NIWA laboratories in Nelson and Hamilton, NZ, respectively.

Wastewater treatment was assessed by changes in ammonia and nitrate concentrations of the wastewater mixture and COD concentrations of the supernatant. Microbial growth was monitored by a combination of Chl *a*, COD, and TSS measurements of the wastewater mixture. Settleability and microalgae incorporation into the biomass were evaluated according to differences between wastewater mixture and supernatant TSS and Chl *a* concentrations.

The biomass was periodically examined using light microscopy (at 1,000 X magnification) to identify the microalgal taxonomy. Bacterial community diversity and abundance data was obtained for single samples of biomass and supernatant by sequencing and analysis of 16S ribosomal RNA (rRNA) gene clone libraries. For a review of this strategy and its applications in microbial ecology see Hugenholtz (2002).

3 RESULTS AND DISCUSSION

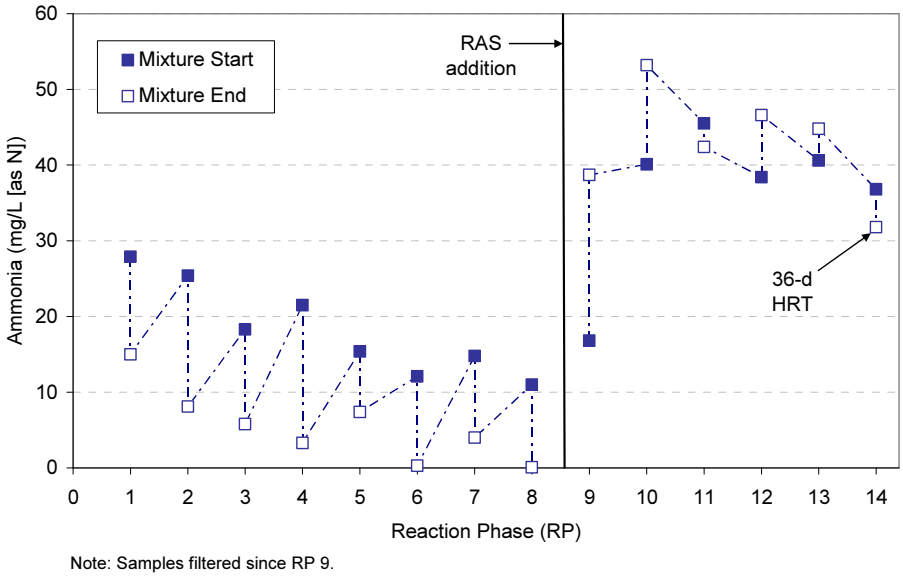
3.1 AMMONIA CONCENTRATIONS

The microbial cultures grew quickly (reaching up to 440 mg/L TSS and 3.7 mg/L Chl *a*) by RP 8 when effluent ammonia averaged 5.5 mg/L (as N), representing a reduction of 46-100% over a 9-day HRT (Figure 2). This wastewater treatment ability by microalgae and bacteria is of key importance to municipal wastewater treatment plants who have difficulty removing ammonia. Measurements of all nitrogen species in the wastewater confirmed that ammonia constituted 88 to 93% of the total nitrogen while nitrate concentrations were less than 3 mg/L and nitrite was not detected (i.e., < 2 mg/L) throughout the study. The nitrogen budget indicates most ammonia was assimilated into biomass and/or volatilised into nitrogen gas by RP 8, when the cultures appeared to reach steady-state growth phase based on TSS concentration.

In an attempt to improve flocculation and settleability of the biomass, 2.5 L of approximately 4-fold concentrated RAS and 4.5 L of primary treated wastewater were added to each SBR prior to RP 9. (The RAS characteristics were summarised in Table 1.) Regular operating conditions (i.e., batch-fed primary effluent for 9-day HRT) resumed following RAS addition. The RAS addition induced anaerobic and highly reducing conditions within the SBRs. The subsequent breakdown and solubilisation of intracellular organic nitrogen (e.g., protein and nucleic acids) and/or exocellular biopolymers (i.e., RAS binding agent) caused elevated ammonia levels (Figure 2). Nitrification was inhibited, and the pH (i.e., typically < 8.0) prevented volatilisation, so ammonia was removed very slowly thereafter by dilution with primary treated wastewater at the start of each RP and through minimal microbial assimilation. Cui (2004) also found that ammonia levels increased following addition of solubilised sludge to a laboratory reactor treating synthetic wastewater

in anoxic conditions, and that mechanical aeration was required to oxidise the ammonia. At the end of the study, RP 14 was extended to 12 days (i.e., 36-day HRT) to observe the wastewater treatment response over a longer period. Ammonia results for RP 14 suggested that microbial assimilation was still occuring to some extent, and that ammonification had decreased.

Figure 2. Ammonia Concentrations of Mixture



Note: Samples filtered since RP 9.

3.2 PH AND DO CONCENTRATIONS

Microalgae used light provided by the 400-W bulbs to photosynthesize during the light period. The resulting increases in pH and DO concentration gradually decreased during the dark period causing the diurnal fluctuations as shown in Figure 3 and summarised in Table 2. In Figure 3, the end of each RP is indicated (subsequent RP start is implied and immediately follows the denoted RP end). Low-level DO detections during RP 14 were due to dilution effects from tap water added to combat evaporation.

Figure 3. pH and DO Readings Collected at 15-min Intervals

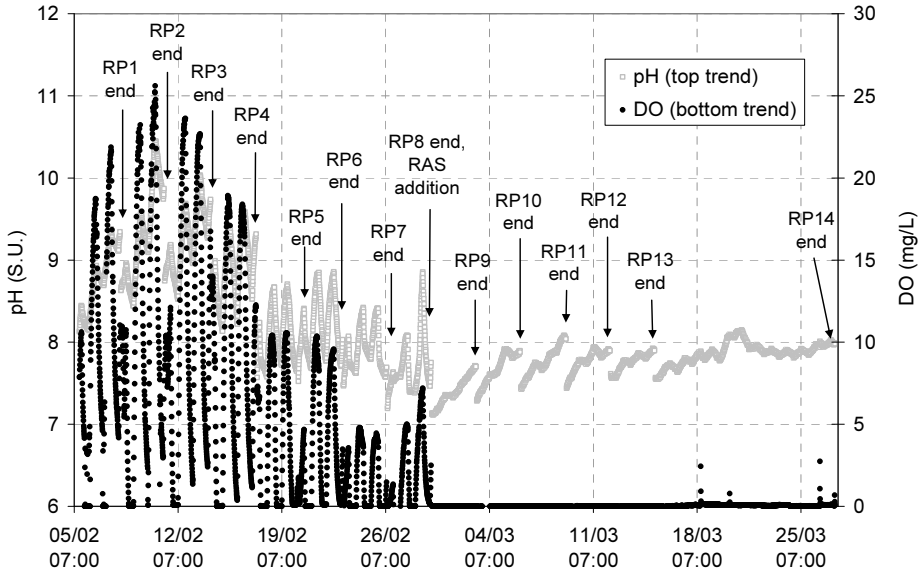


Table 2. Summary of pH and DO Readings Recorded at 15-min Intervals

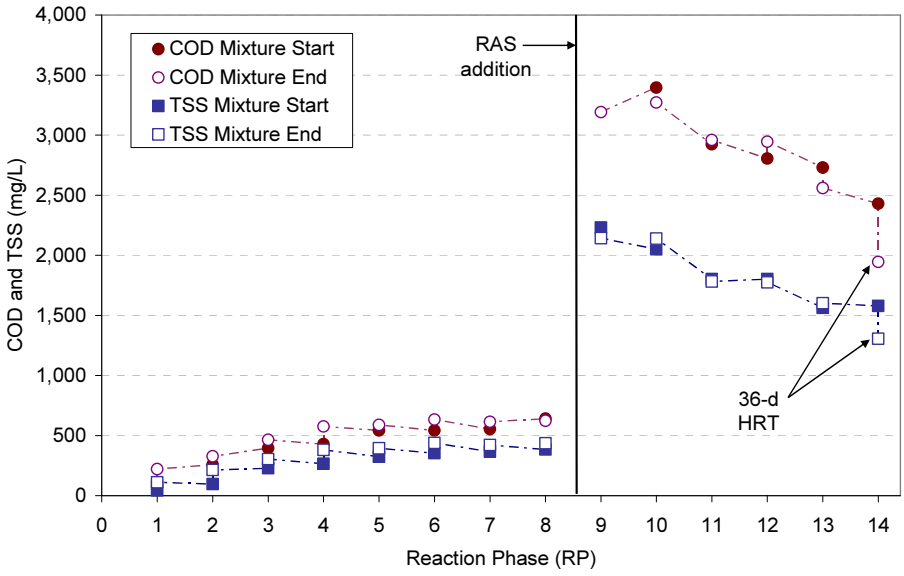
Parameter	Period	Concentration		
		Minimum	Maximum	Mean ± s.d.
pH (S.U.)	Light	7.0	10.5	8.6 ± 0.7
	Dark	7.1	10.4	8.3 ± 0.7
	Light (w/ RAS)	7.0	8.1	7.7 ± 0.2
	Dark (w/ RAS)	6.9	8.1	7.7 ± 0.2
DO (mg/L)	Light	0.0	26	9.2 ± 6.6
	Dark	0.0	24	3.6 ± 4.7
	Light (w/ RAS)	0.0	0.9	0.0 ± 0.0
	Dark (w/ RAS)	0.0	0.1	0.0 ± 0.0

The pH measurements were higher with greater diurnal fluctuations for the non-RAS culture (i.e., 7.0-10.5) compared to the RAS culture (i.e., 6.9-8.1) (Figure 3 and Table 2). This trend was also observed for DO, where levels spanning from 0-26 mg/L became predominantly anaerobic following RAS addition (Figure 3 and Table 2). These differences are presumably due to reduced photosynthesis caused by shading from excess RAS, which would have reduced pH and DO fluctuations. Furthermore, more efficient CO_2/O_2 exchange between microalgae and aerobic bacteria resulting from a higher microbial content in the SBRs after RAS addition would have also minimised fluctuations.

3.3 COD AND TSS CONCENTRATIONS

The COD and TSS concentrations in the wastewater mixture at the end of each RP increased by up to 105 and 152%, respectively, as the suspended microbial biomass grew during RPs 1-8 (Figure 4). Following the initial spike from RAS addition however, COD concentrations at the end of each RP decreased by 5% and TSS by 3% on average, during RPs 9-14. These precipitous concentrations indicated a net reduction of biomass in the mixture most likely due to death of aerobic bacteria.

Figure 4. COD and TSS Concentrations of Wastewater Mixture



The TSS concentrations correlated well with COD, VSS, Chl *a*, and turbidity concentrations as shown in Table 3. These relationships were especially strong for samples measured during RPs 9-14. The R^2 values derived for TSS and COD (0.976-0.990) and TSS and VSS (0.712-0.999) indicate that the cultures were highly carbonaceous and likely predominantly organic (Table 3). These strong correlations could enable some parameters (in future studies) to be calculated by the regression equations in lieu of performing the more time-consuming and expensive suite of analyses.

Table 3. TSS Correlations for Operational Periods

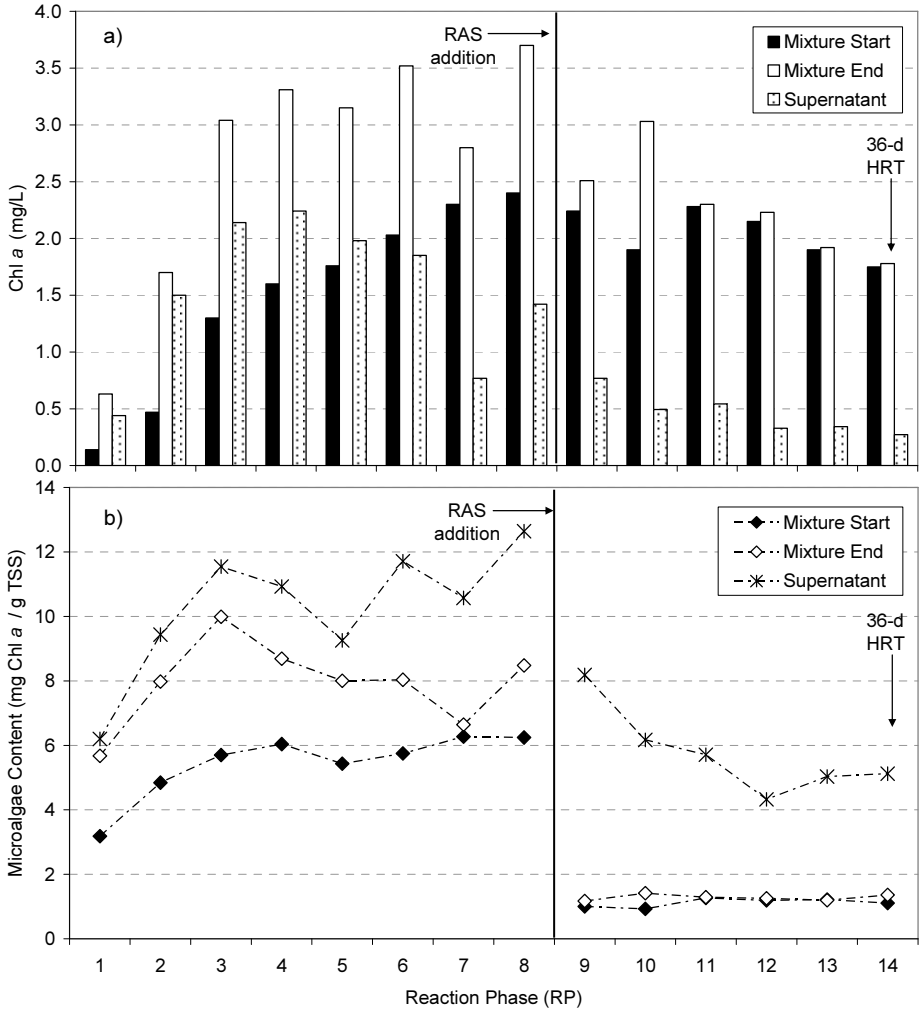
Analyte	Non-RAS Culture (RPs 1-8)		RAS Culture (RPs 9-14)	
	R^2 Value	Equation	R^2 Value	Equation
Chl *a* (µg/L)	0.778	y = 6.9369x + 208.2	0.932	y = 0.9774x + 389.08
COD (mg/L)	0.976	y = 1.3251x + 75.343	0.990	y = 1.4783x + 207.3
Turbidity (NTU)	0.977	y = 0.6314x - 17.653	0.999	y = 0.5092x + 23.485
VSS (mg/L)	0.712	y = 0.7887x - 30.451	0.999	y = 0.8312x - 13.448

Note: y = analyte; x = TSS concentration (mg/L).

3.4 MICROALGAE GROWTH AND SETTLEABILITY

The Chl *a* concentration and microalgae content (mg Chl *a*/g TSS) of the biomass directly indicated microalgae growth (Figure 5). For RPs 1 and 2, Chl *a* of the supernatant and wastewater mixture end were similar (Figure 5a), because rapid growth caused the microalgae to be unsettleable. Microalgae exhibit a high negative surface charge during exponential growth, which keeps them separated them by repulsive forces (Becker 1994; Henderson et al. 2008), thereby impeding aggregation and settling. As the culture aged and the growth rate and cell surface charge decreased, there was more pronounced difference in Chl *a* concentration between supernatant and wastewater mixture end (Figure 5a). To account for these differences after RP 2, it is likely that spontaneous clumping and settling, or bioflocculation, of the microalgae occurred.

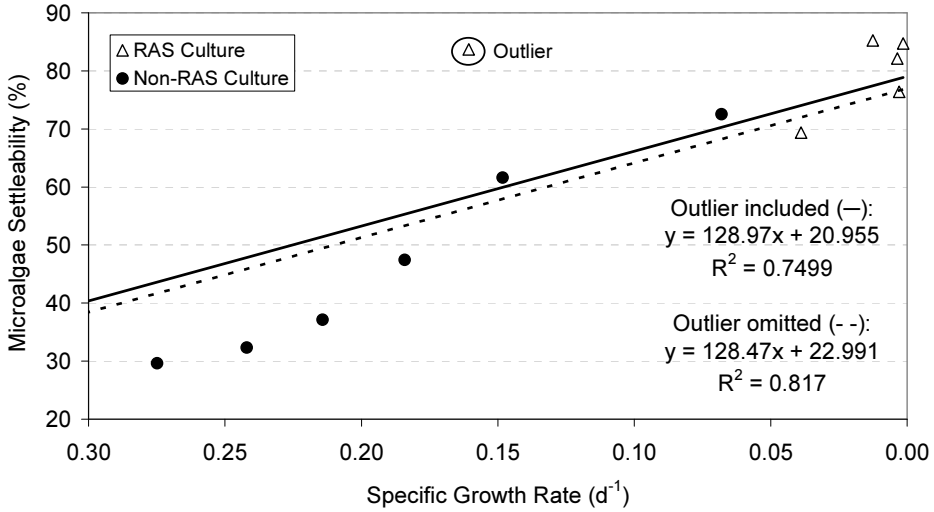
Figure 5. Microalgae Growth: a) Chl a Concentration and b) Microalgae Content of the Biomass



Microalgae growth was severely inhibited following the RAS addition, with minimal growth observed between wastewater mixture start and end in RPs 9-14 as indicated by Chl *a* concentration differences (Figure 5a). These inhibiting conditions reduced average wastewater mixture end Chl *a* from 3.3 to 2.3 mg/L (Figure 5a) and microalgae content of the biomass from 8.3 to 1.3 mg Chl *a*/g TSS (Figure 5b) from RPs 3-8 to 9-14. Ammonia concentrations greater than 28 mg/L (as N) have been shown to impair microalgae growth and photosynthesis at pH above 8.0 (Abeliovich and Azov 1976). Following RAS addition, ammonia concentrations were 32-53 mg/L (as N) (Figure 2) and pH levels were 6.9-8.1 (Figure 3). The RAS may have caused some ammonia toxicity even though the average pH was only 7.7. In addition, significant shading of the microalgae by RAS reduced photosynthesis potential by limiting light penetration into the SBRs. Microalgae concentration in the supernatant biomass was greater than the wastewater mixture biomass (Figure 5b) indicating that they were less settleable than sediment and other suspended constituents of the primary treated wastewater and RAS present in the mixture.

Bioflocculation was evident from the substantial supernatant and wastewater mixture end Chl *a* concentration differences observed from RP 3 onwards (Figure 5a). In support of this phenomenon, a strong linear relationship (75-82%) existed between microalgae settleability and specific growth rate (Figure 6). In Figure 6, settleability was calculated as the the percent of Chl *a* concentration settled from the supernatant at mixture end, while specific growth rate was calculated based on the increase in Chl *a* concentration during each RP. Clearly, settleability improved as growth rate of the culture decreased as attributed to reduced negative cell surface charge allowing bioflocculation.

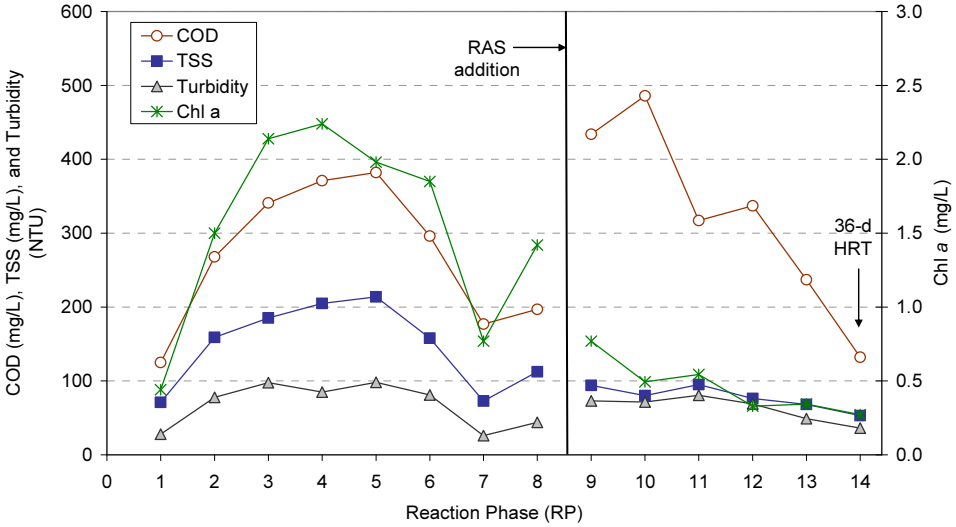
Figure 6. Effect of Growth Rate on Microalgae Settleability



3.5 SUPERNATANT WASTEWATER QUALITY

In addition to Chl *a* (as discussed previously), COD, TSS, and turbidity concentrations of the supernatant were also influenced by biomass settleability during RPs 1-8 (Figure 7). Following RAS addition, the supernatant quality noticeably improved in terms of Chl *a* and TSS concentrations. Unsettleable solids were reduced from 112 to 53-68 mg TSS/L and the amount of suspended microalgae from 1.4 to 0.22-0.34 mg Chl *a*/L (Figure 7). These trends indicate that the RAS was highly settleable within the 1-hr settling periods of the RPs. The calculated SVI for the 30-min settled volume was 140-200 mL/g (data not graphed). The RAS introduced additional flocculating bacteria adapted to the activated sludge process, which presumably produced a greater amount of extracellular biopolymeric flocculants (EBFs). These EBFs are produced naturally by microalgae and bacteria and would have enhanced bioflocculation and, thus, settleability of the biomass (Salehizadeh and Shojaosadati 2001). The COD concentration spiked for RP 9 due to the solubilisation of RAS from anaerobic conditions, but it approached more typical levels of 132-237 mg/L as the culture began to stabilise. Turbidity was comparable irrespective of RAS addition (Figure 7).

Figure 7. Supernatant Characteristics



3.6 RELATED WORK

The experimental approach adopted in this study was similar to that of Gutzeit et al. (2005) who combined *Chlorella vulgaris* and added activated sludge to form microalgal-bacterial flocs. However, a much higher PAR level of 2,000 µmol $m^{-2}s^{-1}$ beget higher microalgae and DO concentrations of 15-37 mg Chl *a*/L and 0.2-1.5 mg DO/L, respectively, within their lab-scale reactor. Their reactor was also operated with a much lower HRT of 1-3 days which would have facilitated dilution of any excess solubilised sludge and may have enabled a quicker culture stabilisation time of 4 weeks following the RAS addition. Feed water characteristics were similar between both studies. In a larger pilot-scale reactor with a 5-7 d HRT and comparable PAR, the supernatant contained 18 mg TSS/L and 0.22 mg Chl *a*/L (Gutzeit et al. 2005). These values are comparable to the final supernatant concentrations of the SBRs in this study (i.e., 68 mg TSS/L and 0.34 mg Chl *a*/L after 9-d HRT and 53 mg TSS/L and 0.27 mg Chl *a*/L after 36-d HRT). These findings support the well-accepted notion that HRT, PAR, and/or microbial species are determing factors in biomass productivity and concurrent wastewater treatment.

3.7 MICROBIOLOGY

3.7.1 MICROALGAE

The microalgal community changed over the course of the study in response to environmental conditions. The Pond 6 wastewater inoculum was dominated by green microalgae including *Micractinium* sp., *Pediastrum* sp., *Lepocinclis* cf. *texta*, and unknown species (i.e., cf. *Chlorella* sp. and/or *Choricystis* sp.). After operating the SBRs for approximately 2 weeks in batch-fed mode, the microalgae community was re-examined under the 1,000 X microscope. Green microalgae continued to dominate, and *Scenedesmus* sp. and *Schroederia robusta* were abundant in addition to the taxa identified in the original inoculum. Approximately 3 weeks following the RAS addition, only diatoms, *Pediastrum* sp., and unknowns (i.e., cf. *Chlorella* sp. and/or *Choricystis* sp.) were found. Pond 6 sampling results by Christchurch City Council also suggest that *Micractinium* sp. and other taxa are more sensitive to wastewater quality than *Chlorella* sp. and/or *Choricystis* sp. (Novis 2007). In addition, Tarlan et al. (2002) found that diatoms became more abundant than green algae at lower light intensity and higher COD loading of paper industry wastewater. Therefore, the RAS presumably caused the decrease in green microalgae diversity due to ammonia toxicity, bacterial shading, and higher organic loading, which enabled the proliferation of diatoms.

3.7.2 BACTERIA

A culture-independent 16S rRNA analysis of the bacterial community structure was performed on samples collected at the end of the study. The bacterial community within the settled biomass was dominated by members of the *Firmicutes* (45%) and *Proteobacteria* (39%) phyla. Anaerobic species, notably members of the genus *Clostridium* accounted for 80% of the *Firmicutes*, while the *Proteobacteria* were dominated by the sulphur oxidiser *Thiothrix* (47% of the *Proteobacteria*). These results are consistent with the notion that

anaerobic conditions occurred after the RAS addition. This microbial research is ongoing; further data will be presented in due course.

4 SUMMARY AND CONCLUSIONS

- Microbial (microalgal-bacterial) biomass was grown in batch-fed SBRs on a mixture of oxidation pond and primary treated wastewaters from CWTP with effective ammonia removal.
- Addition of RAS to enhance biomass settling rates increased ammonia, COD, and TSS concentrations of the wastewater mixture initially. Therefore, effective wastewater treatment with this amount of RAS has not yet been demonstrated.
- The RAS addition improved the settleability of the biomass by reducing unsettleable solids and suspended microalgae in the supernatant. This finding suggests that the biomass may be more harvestable for biofuel production or other uses compared to non-RAS cultures.
- The RAS addition caused a decrease in microalgae growth and diversity possibly due to ammonia toxicity, shading, and/or higher organic loading.
- Microalgae could not provide adequate photosynthetically produced DO to wholly sustain bacterial respiration after the RAS addition. This imbalance caused a loss of biomass due to solubilisation of organic matter and/or aerobic microbial death. The predominance of *Clostridium* spp in the final biomass is consistent with a shift in bacterial community structure to favour anaerobic species following RAS addition. A smaller addition of RAS may have caused less detrimental effects on the microbial community and maintained a greater balance in the microalgal-bacterial symbiosis.
- Bioflocculation and incorporation of microalgae into the RAS flocs were the primary mechanisms affecting settleability. A good linear relationship existed between specific growth rate of the microalgae and biomass settleability. As microalgae aged and culture density increased, decreasing growth rates positively impacted settleability via bioflocculation. This trend indicates that growth conditions can be manipulated to obtain a more harvestable biomass.
- Strong linear correlations between TSS and COD and TSS and VSS indicated that the culture was carbonaceous and probably predominantly organic, while TSS and Chl *a* were also strongly correlated indicating a photosynthetic relationship. These measurements can be employed as more cost-effective and less time-consuming surrogate indicators for future studies.

5 FUTURE WORK

This preliminary study raised several research questions regarding the growth and settleability of microalgae to continuous-fed primary treated effluent (both with and without RAS inoculum) compared to secondary treated effluent in Christchurch, NZ. Mean summer and winter conditions (e.g., air temperature, PAR, day length, and humidity) for Christchurch will be simulated in the laboratory to determine impacts on biomass productivity, settleability, and composition. The research will evaluate prospective feed wastewaters for the purpose of microalgal biofuel production and provide a better understanding of their potential merits or limitations for larger scale operation. These results will guide the design of pilot-scale field studies employing high-rate algal ponds.

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