Microbial (Microalgal-Bacterial) Biomass Grown on Municipal Wastewater for Sustainable Biofuel Production

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- Earl Denman (1954)

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Terminology

Abbreviations and Acronyms

Al	aluminium
ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
AP	primary wastewater culture with P6 and AS inocula (Ch. 4)
APA	American Psychological Association
APHA	American Public Health Association
AS	activated sludge
ASP	Aquatic Species Program
$bCOD_o$	initial total biodegradable chemical oxygen demand
BOD	biochemical oxygen demand
C	carbon
C/N	carbon to nitrogen ratio
Ca	calcium
CCC	Christchurch City Council
cf.	comparable to
CH_4	methane
Chl *a*	chlorophyll *a*
Co	cobalt
CO	tap water (control) culture with P6 inoculum (Ch. 4)
CO_2	carbon dioxide
CO_3^{2-}	carbonate
COD	chemical oxygen demand
CRC	Canterbury Regional Council
CSTR	continuous stirred tank reactor
Cu	copper
CWTP	Christchurch Wastewater Treatment Plant
D50	50% diluted SBR culture (Section 3.3.4)
D75	75% diluted SBR culture (Section 3.3.4)
DAF	dissolved air flotation
DI	deionised water
DIC	dissolved inorganic carbon
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DOE	(U.S.) Department of Energy
dw	dry weight
EBF	extracellular biopolymeric flocculants
EPA	(U.S.) Environmental Protection Agency
EPS	extracellular polymeric substances
F/M	food to microorganism ratio
FAME	fatty acid methyl ester
Fe	iron
GHG	greenhouse gas
H	hydrogen
H_2O	water
HC	heat of combustion

HCO_3^-	bicarbonate
HRAP	high-rate algal pond
HRT	hydraulic retention time
IDF	International Dairy Federation
K	potassium
K_s	half-saturation constant
LCA	life cycle assessment
LMM	linear mixed-effects model
MCL	maximum contaminant level
Mg	magnesium
Mn	manganese
MUFA	monounsaturated fatty acid
N	nitrogen
Na	sodium
NA	not available
NH_3	ammonia
NH_4^+	ammonium
NIWA	National Institute of Water and Atmospheric Research
NO_2^-	nitrite
NO_3^-	nitrate
NTU	nephlemetric turbidity units
NZ	New Zealand
O_2	oxygen
OH^-	hydroxide
P	phosphorus
P1	(CWTP Oxidation) Pond 1
P6	(CWTP Oxidation) Pond 6
PAR	photosynthetically active radiation
PBR	photobioreactor
PCR	polymerase chain reaction
PE	primary wastewater culture with P6 inoculum (Ch. 4)
PO_4^{3-}	phosphate
PQL	practical quantitation limit
PUFA	polyunsaturated fatty acid
QA	quality assurance
QC	quality control
R	removal efficiency
R1/R2	ambient climate CSTR cultures (Section 3.2)
RDP	Ribosomal Database Project
RNA	ribonucleic acid
RP	(SBR) reaction phase
RPD	relative percent difference
rpm	revolutions per minute
S	sulphur
S4	4-d SRT, primary wastewater culture with P6 and AS inocula (Ch. 5)
S8	8-d SRT, primary wastewater culture with P6 and AS inocula (Ch. 5)

S12	12-d SRT, primary wastewater culture with P6 and AS inocula (Ch. 5)
sBOD	soluble biochemical oxygen demand
SBR	sequencing batch reactor
SBR1/2	ambient climate SBR cultures with P6 inoculum (Section 3.3)
sCOD	soluble COD
$sCOD_i$	influent soluble COD
$sCOD_t$	effluent soluble COD
SE	secondary wastewater culture with P6 inoculum (Ch. 4)
SFA	saturated fatty acid
Si	silicon
SM	standard method
SO_4	sulphate
sp.	one species
spp.	multiple species
SRT	solids retention time
SV	settled volume
TC	total carbon
tCOD	total chemical oxygen demand
$tCOD_i$	influent total chemical oxygen demand
$tCOD_o$	initial total chemical oxygen demand
TKN	total Kjeldahl nitrogen
TN	total nitrogen
TOC	total organic carbon
TP	total phosphorus
TS	total solids
TSS	total suspended solids
UC	University of Canterbury
v/v	volume per volume
VFA	volatile fatty acid
VS	volatile solids
VSS	volatile suspended solids
WTP	wastewater treatment plant
Zn	zinc
μ	specific growth rate
μ_m	maximum specific growth rate

Units

d	days
EJ	exajoules
g	grams
Gt	gigatonnes
ha	hectares
hp	horsepower
h	hours
kg	kilograms
Kt	kilotonnes
L	litres
m	meters
mg	milligrams

min	minutes
mL	millilitres
Mt	megatonnes
mV	millivolts
nm	nanometers
SU	standard pH units
t	tonnes
µm	micrometer
µmol	micro mole

Statistics

M	mean
df	degrees of freedom
F	*F* statistic (using ANOVA)
n	number of replicates/samples
p	significance level
r	Pearson's correlation coefficient
SD	standard deviation
t	*t* statistic

Abstract

High biomass productivity and efficient harvesting are currently recognised challenges in microbial biofuel applications that were addressed by this research using ecological engineering principles and an integrated systems approach. Microbial (microalgal-bacterial) biomass was grown in laboratory reactors using municipal wastewaters from the Christchurch Wastewater Treatment Plant (CWTP) in New Zealand. Reactors were inoculated with native microbes, fed with primary and secondary treated wastewaters, and subjected to various hydraulic and solids retention times (i.e., 1.4- to 9-d HRT and 4- to 80-d SRT, respectively) under cold, warm, and ambient climate conditions. Biomass settleability and productivity (i.e., settleable productivity) were sequentially improved over the course of experiments to optimise settleable productivity at 21 $g/m^2/d$ on average using primary treated wastewater, 2-d HRT, 12-d SRT, and warm climatic conditions. Secondary treated wastewater was a poor substrate most likely because of low C, elevated pH, and supersaturated oxygen levels limiting growth. Biomass recycling generally improved settleable productivity of primary treated wastewater cultures since productivity increased at short HRT and settleability increased at longer SRT. No overriding trends were found relating productivity or settleability to biomass ecology or biochemistry.

Growth rate modelling of warm climate cultures indicated that heterotrophy was mostly C limited at long (≥ 4-d) HRT and DO limited at short (≤ 2-d) HRT of primary treated wastewater while photoautotrophy was probably always light limited. Nevertheless, almost 50% greater C fixation was achieved using these systems compared to conventional activated sludge systems. Cold climate cultures, with up to 66% less biomass than warm climate cultures, were limited by lower light and/or temperature (i.e., 13 °C mean water temperature with 410 $\mu mol/m^2/s$ photosynthetically active radiation [PAR] for 9.6 h/d vs. 21 °C mean water temperature with 925 $\mu mol/m^2/s$ PAR for 14.7 h/d).

Biomass settleability was facilitated by microbial aggregation into stable, compact flocs over time and also by bioflocculation during 1-h sedimentation periods. These mechanisms were largely influenced by wastewater loading and microbial growth rate, but also to a lesser extent by monitoring methods (i.e., light, duration, and sedimentation container). Settleability of primary treated wastewater cultures was mainly greater than 70% and more consistent when operated at longer SRT and shorter HRT compared to only 22% on average for secondary treated wastewater cultures.

Symbiotic growth of native microalgae and bacteria promoted efficient O_2/CO_2 exchange to improve productivity and enhanced natural floc formation to improve settleability while requiring low energy inputs and providing some wastewater treatment. These capabilities

greatly increased the biomass' sustainability for biofuel production compared to other feedstocks. This research demonstrated the value of biomass recycling to concurrently achieve greatest productivity and settleability to maximise harvestable yield since the overall growth rate of more total biomass was reduced at longer SRT which thereby facilitated excellent floc formation and sedimentation at shorter HRT. The resulting biomass was best suited for biofuel conversion pathways such as anaerobic digestion or thermochemical liquefaction. Potential other uses included animal feed and fertiliser since biomass was harvested without additional chemicals.

Research Publications

Peer-Reviewed Conference Proceedings

Valigore, J. M., O'Sullivan, A. D., and Gostomski, P. (2009). "Municipal Wastewater Selection for Microbial Biodiesel Production." 8th International Conference on Sustainable Energy Technologies, Aachen, Germany, 6 pp.

Valigore, J. M., Turner, S., and O'Sullivan, A. D. (2008). "Microbial Biomass Grown on Primary Treated Wastewater." NZWWA's 50th Anniversary Conference and Expo, New Zealand Water and Wastes Association, Christchurch, NZ, 10 pp.

Conference Abstracts and Posters

Valigore, J. M., Gostomski, P., and O'Sullivan, A. D. (2010). "Effect of Hydraulic and Solids Retention Times on Growth and Settleability of Microalgal-Bacterial Biomass for Biofuel Production." Presented at (1) 32nd Symposium on Biotechnology for Fuels and Chemicals, Clearwater Beach, FL, USA; (2) New Zealand Society of Chemical Engineers (SCENZ-IChemE) AGM and Conference, Christchurch, NZ; and (3) College of Engineering Research Conference, University of Canterbury, Christchurch, NZ.

Turner, S. J., Biswas, K., Valigore, J. M., and O'Sullivan, A. D. (2008). "Growth of Microalgal-Bacterial Biomass on Primary Treated Wastewater." New Zealand Microbiology Society Conference, Christchurch, NZ.

Valigore, J. M., and O'Sullivan, A. D. (2008). "Are Algae the Answer? Wastewater Treatment and Biofuel Production in Christchurch." College of Engineering, University of Canterbury, Christchurch, NZ.

Unpublished Reports

Valigore, J. M. (2009). "Separation of Activated Sludge and Oxidation Pond 6 Microalgae at Christchurch Wastewater Treatment Plant." Christchurch City Council, Christchurch, NZ, 6 pp.

Valigore, J. M. (2008). "Microalgal-Bacterial Biomass for Coupled Biofuel Production and Wastewater Treatment." PhD Research Proposal, Department of Civil and Natural Resources Engineering, University of Canterbury, Christchurch, NZ, 15 pp.

Chapter 1: Introduction

1.1 Background

> *The security of people and nations rests on four pillars - food, energy, water and climate. They are all closely related, and all under increasing stress.*
> - Tom Burke (2008)

Global demand for transport and industrial fuels is increasing, but known oil reserves are diminishing, so a serious imbalance of demand and supply is imminent for conventional fuels. Worldwide energy consumption is projected to increase by 49% from 522 exajoules (EJ) in 2007 to 780 EJ in 2035 (U.S. DOE 2010). Liquids will remain key fuel sources at 236 EJ or 30% of energy through 2035 (Figure 1-1) due to their importance in transportation and industry, but their relative contribution will decrease due to anticipated rising crude oil prices. Meanwhile, it is well accepted that global warming increases in response to greenhouse gas (GHG) emissions (Intergovernmental Panel on Climate Change 2007) and so, the need to identify more sustainable and environmentally acceptable sources of energy is paramount. Renewable energies including water, wind, solar, geothermal, and especially biofuels have received much political support recently to help solve this problem. In New Zealand (NZ) in 2007, about one-half of energy needs were met by imported oil and petroleum products, which were primarily used in the transportation sector (Ministry of Economic Development 2007). The government set targets including 90% renewable electricity generation by 2025, 50% reduction of net carbon

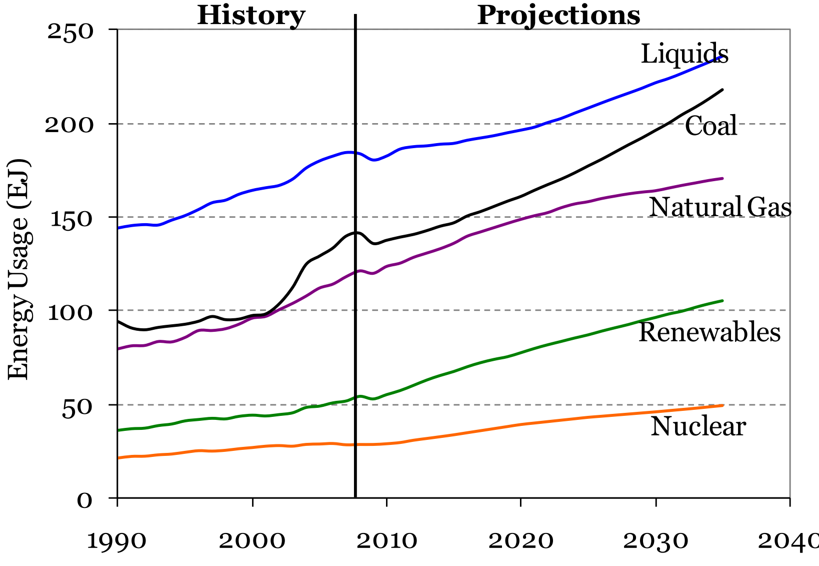

Figure 1-1. Recent Past and Projected Worldwide Marketed Energy Usage (U.S. DOE 2010).

emissions (compared to 1990 levels) by 2050, and an emissions trading scheme to encourage reduction of GHGs and more efficient energy use in order to meet obligations of the Kyoto Protocol (Ministry of Economic Development 2010). Renewable energies are expected to increase to 14% of total worldwide energy consumption by 2035 (Figure 1-1) from a combination of rising oil costs, environmental concerns, and government initiatives to promote sustainable practices (U.S. DOE 2010).

1.2 Conventional Biofuels

Biofuels are solid, liquid, and gaseous substances derived from virgin or waste biomass that can be combusted to provide energy. They are very attractive alternatives to conventional fuels. When derived from domestic sources, biofuels can offer numerous benefits over petroleum or coal-based fuels. They can contribute to energy supply security through reduced dependence on imported fossil fuels and may catalyse new economic development and growth as the biofuels' industry advances. Additionally, biofuel production can demonstrate environmental stewardship by relying on natural carbon cycling. It can offset carbon dioxide (CO_2) emissions by being less energy-intensive to acquire than fossil fuels. For example, utilising 100 Kt sawmill waste residues for energy could displace 205 Kt CO_2 emissions from fossil fuels by saving transport, processing, and other energies (Cowie and Gardner 2007). Depending on biomass production practices and their conversion efficiencies, 4 to 16 Gt/yr CO_2 emissions could be mitigated through worldwide replacement of fossil fuels with biofuels (Sampson et al. 1993).

There is currently intense debate about the use of land-based crop biofuels, which can have negative ecological and social impacts in their production (Reijnders 2006; Crutzen et al. 2007; Lovett 2007; Fargione et al. 2008; Davis et al. 2009). For example, sugarcane ethanol has led to destruction of rainforests in Brazil and food shortages by replacement with fuel crops (Goldemberg et al. 2008). All aspects of sustainability must be considered—economical, environmental, and social impacts—to assess a biofuel's true value (or cost). Various tools have emerged recently to gauge sustainability including life cycle assessments (LCAs) (Davis et al. 2009; Stephenson et al. 2010), embodied energy (or emergy) (Brown et al. 2008), ecological footprint (Stoeglehner and Narodoslawsky 2009), and triple bottom line accounting (Hacking and Guthrie 2008). These theoretical outcomes, however, can be highly subjective depending on assumptions and available data, but sensitivity analyses can reduce uncertainties.

1.3 Sustainable Biofuels from Microalgae

Microalgal biomass grown on wastewater has potential for sustainable biofuel production (Van Harmelen and Oonk 2006; Chisti 2007; Pittman et al. 2011). A wide range of biofuels can be produced from microalgal biomass (Demirbas 2010) to provide an effective partial solution to the global energy challenge outlined earlier. In comparison to other biofuel feedstocks (e.g., rapeseed, maize, etc.), microalgal biomass can offer many advantages including:

- high lipid content (precursor of biodiesel) and productivity (Sheehan et al. 1998),
- less area required compared to land-based crops (Chisti 2007),
- ability to recycle nutrients from unconventional water resources (e.g., wastewater and brackish water including oceans) (Benemann et al. 2003b; Sreesai and Pakpain 2007),
- ability to capture CO_2 from flue gas emissions and other enriched sources (Yun et al. 1997; Benemann et al. 2003b; Van Harmelen and Oonk 2006),
- potential for continuous production throughout the year depending on climate (Van Harmelen and Oonk 2006),
- free of lignin and other large biopolymers (found in woody biomass) that may interfere with biomass processing and conversion (Alvira et al. 2010),
- valuable co-products such as fertiliser and animal feed (Benemann et al. 2003b; Van Harmelen and Oonk 2006; Harun et al. 2010), and
- affordable and fast research and development due to rapid growth rates (Benemann and Oswald 1996).

Biofuel production from microalgae has been researched at a varying intensity since the 1950s. Initial research focused on anaerobic digestion of microalgae to produce methane (CH_4). Oswald and Golueke (1960) envisioned a sustainable system that used CH_4 from the digestion process to generate electricity. Additionally, they proposed using municipal wastes (e.g., sewage), CO_2 from power plant emissions, and residual biomass from the digestion process to sustain new microalgal growth. This concept and the first oil crisis of the 1970s led to concurrent wastewater treatment and biofuel production studies using microalgae.

From about 1980 to 1996, the U.S. Department of Energy (DOE) funded the Aquatic Species Program (ASP) through the National Renewable Energy Laboratory in Golden, Colorado, USA. This project aimed to assess the feasibility of biodiesel production from high lipid-content microalgae. Major accomplishments of the ASP included a unique microalgae collection for research purposes, improved understanding of microalgae physiology and biochemistry, and advancements in molecular biology and genetic engineering of microalgae (Sheehan et al. 1998). The authors concluded that large-scale biodiesel production from microalgae was unfavourable for stand-alone production at the time, but that integration of co-processes such as wastewater treatment could improve its economic and environmental outlook.

In 2002, the International Network for Biofixation of CO_2 and Greenhouse Gas Abatement with Microalgae was formed to guide the development of projects focused on converting GHGs into microalgal biomass (Benemann et al. 2003a). Key aspects were identified that required further research: 1) strain selection and maintenance, 2) genetic engineering, 3) physiology, 4) inoculum production, 5) culture stability, 6) productivity, 7) harvesting, 8) conversion

processes, 9) co-products and co-processes, and 10) engineering designs. In addition to promoting research into these areas, the practical potential of microalgae for CO_2 abatement was investigated. Results from this initiative estimated that treatment of municipal, dairy, and swine wastes using microalgae could mitigate 90 Mt/yr CO_2 (Van Harmelen and Oonk 2006). Co-production of biofuels and/or fertilisers would further increase mitigation potential by producing renewable energy and reducing fossil fuel usage.

More recently, several companies have been making technological advances in microalgal biotechnology, but only a few have produced biofuel from microalgae. A comprehensive survey of microalgae producers worldwide was compiled by Edwards (2009) and summarised by Singh and Gu (2010). Within NZ, Aquaflow Bionomic Corporation claimed to have produced the first known sample of biodiesel from microalgae grown in municipal wastewater (Kiong 2006). Additionally, the National Institute of Water and Atmospheric Research (NIWA; a NZ Crown Research Institute) has conducted research incorporating high-rate algal ponds (HRAPs) into wastewater treatment trains for the primary purpose of improving water quality (Craggs 2001; Craggs et al. 2003). (HRAPs are discussed in Section 1.4.6.1.) These treatment trains include an enhanced facultative pond, HRAP, settling pond, and maturation pond in series. Energy can be recovered during the treatment process by capturing biogas from the facultative pond or from the digestion of microalgal biomass for combustion. Recycling the flue gas to the HRAP could also potentially increase biomass productivity and wastewater treatment (Heubeck et al. 2007). More recently in 2009, NIWA joined forces with Solray Energy, who specialises in bio-oil conversion, to launch a 5-ha demonstration HRAP incorporating CO_2 addition for biomass production at Christchurch Wastewater Treatment Plant (CWTP) (NIWA 2009).

1.4 Microbial Biomass Consortia

Much of the microalgal research to date has not investigated the involvement of other microbes in microalgal production systems. Although such photosynthetically-driven systems are usually dominated by microalgae, numerous bacteria, archaeans, fungi, protozoa, and microscopic invertebrate animals (e.g., rotifers and nematodes) may also be present, especially in open systems, forming a stable microbial community. The inoculum(s) and growth environment ultimately dictate how the communities will self-organise in the biomass production systems. Microalgae and bacteria are the focus of this research due to their symbiotic relationship (Section 1.4.4) and ability to treat wastewater (Section 1.4.5) for the sustainable production of microbial (microalgal-bacterial) biomass.

1.4.1 Microalgae

Microalgae are oxygenic, photosynthetic microorganisms comprising many of the eukaryotic algae and cyanobacteria (Graham et al. 2009). Microalgae are very diverse in morphology (e.g., motile and non-motile unicells and colonies, unbranched and branched filaments), cytology

(e.g., eyespot presence or absence, cell wall structure, flagella number and length), and reproduction characteristics (e.g., type of sexual life cycle, morphology of asexual spores). They are generally classified according to their colour/pigments, storage products, cell covering, and organelles as described in the literature (South and Whittick 1987; Graham et al. 2009). Classes of microalgae globally common in municipal wastewaters include Chlorophyceae (green algae), Euglenophyceae (euglenoids), Bacillariophyceae (diatoms), and Cyanophyceae (blue-green algae) (Craggs 2001; Garden 2005; Novis 2007; Wiltshire and Broady 2008; Graham et al. 2009) (Table 1-1). These free-floating microalgae (or phytoplankton) can range in size from < 5 µm (e.g., *Chlorella* sp.) up to 500 µm (e.g., *Volvox* sp.) (South and Whittick 1987) and can double their populations more than twice daily (Graham et al. 2009) highlighting their diversity and growth potential.

Table 1-1. Microalgae Classes Commonly Found in Municipal Wastewater.

Common Name	Cell Type and Class	Example Specimen	Photosynthetic Pigments	Cell Envelope	Flagella
Green microalgae	Eukaryote: Chlorophyceae	*Scenedesmus* sp.	Chl *a*, Chl *b*, β- and other carotenes, xanthophylls	Usually scaled, naked, or cellulose wall	Normally two of equal length
Euglenoids	Eukaryote: Euglenophyceae	*Lepocinclis* sp.	Chl *a*, Chl *b*, β- and other carotenes, xanthophylls	Proteinaceous strips beneath plasma membrane	Normally two, often of unequal length
Diatoms	Eukaryote: Bacillariophyceae	*Nitzschia* sp.	Chl *a*, Chl *c*, β-carotene, xanthophylls	Usually silica wall	One for male gametes
Cyanobacteria or blue-green microalgae	Prokaryote: Cyanophyceae	*Oscillatoria* sp.	Chl *a*, phycocyanin, allophycocyanin, phycoerythrin, β-carotene, xanthophylls	Peptidoglycan	Absent

(Source: Graham et al. 2009)

Microalgae can be identified by genus using light microscopy at magnifications up to 2,000 X depending on taxonomic knowledge. Molecular genetics (e.g., comparison of nucleotide sequences of particular genes) is increasingly used to define taxa (Graham et al. 2009); however, these tools are not yet well-suited for characterising unknown and multi-species cultures since they are limited to those species that have been previously defined in genomic databases. Hence, traditional light microscopy can more effectively estimate sample diversity, especially for mixed microalgal cultures present in the wastewater examined during this research while also

identifying species that may affect process parameters. For example, *Chlorella* spp. are small (≤ 5 µm), spherical microalgae that can be difficult to harvest (Becker 1994).

1.4.2 Bacteria

Bacteria are exclusively prokaryotic cells surrounded by a rigid cell wall, and they mostly occur in three shapes: spheres (cocci), spirals (spirilla), and rods (bacilli). Generally, cells are 1-10 pg in mass and 1-2 µm in diameter (Mara and Horan 2003). Bacteria exist as unicells, pairs, chains, and clusters, and they can be motile or non-motile. Genera common to aerobic municipal wastewater include *Zooglea*, *Pseudomonas*, *Chromobacter*, *Achromobacter*, *Alcaligenes*, and *Flavobacterium* (Mara and Horan 2003). Under optimal growth conditions, some bacteria can double their populations through asexual reproduction in < 30 min, which makes them the fastest growing organisms known (Stephenson and Judd 2002).

Cell structure characteristics, reaction to the differential Gram stain, and metabolism are of limited use in bacterial classification. Within the past two decades, bacteria have been increasingly identified using molecular methods, which circumvent the need for their laboratory cultivation (Gilbride et al. 2006). These molecular techniques can enable researchers to examine the diversity and dynamics of bacterial communities and investigate how they affect process parameters such as settleability. For example, *Microthrix parvicella* and *Haliscomenbacter hydrosis* can cause poor settleability and loose compaction of activated sludge (AS) under nutrient limitation (Rittmann and McCarty 2001) which are problematic for wastewater treatment plants (WTPs). Conversely, inoculating cultures with identified floc-formers such as *Citromonas*, *Flavobacterium*, and *Zooglea* (Gerardi 2006) could improve harvesting of microbial biomass without the need for (chemical) flocculants. Floc-forming bacteria are of interest in this research due to their potential to enhance biomass harvestability.

1.4.3 Microbial Nutrition

Microorganisms have a wide range of metabolic strategies as detailed elsewhere (Richmond 2004; Campbell and Reece 2008). Generally, bacteria found in municipal wastewater are chemoheterotrophs and microalgae are photoautotrophs. Together, they can form a symbiotic relationship where microalgae source CO_2 and inorganic nutrients from bacterial metabolism byproducts, and bacteria source O_2 from microalgal photosynthesis (McGriff and McKinney 1972; Eisenberg 1981; Toerien et al. 1984; Gutzeit et al. 2005; Munoz and Guieysse 2006). In addition to these metabolic requirements, the microbes also require N and P plus other elements (e.g., S, K, Fe, Ca, Mn, Cu) in smaller quantities for growth and cellular functions depending on the species (South and Whittick 1987; Madigan et al. 2000). For example, diatoms also require silicon (Si), a major component of their cell wall. Overall, metabolic and nutritional needs of the microbes can be met through microalgal-bacterial symbiosis, the atmosphere, and the growth medium.

1.4.4 Microbial (Microalgal-Bacterial) Floc Dynamics and Characteristics

Due to their symbiosis, photoautotrophic microalgae and chemoheterotrophic bacteria often co-aggregate (along with other microbes) into compact flocs, which thereby enhances CO_2 and O_2 exchange and hence their coupled growth (Munoz and Guieysse 2006). Microalgae and bacteria may also affect each other's growth by secreting extracellular biopolymeric flocculants (EBFs) (Eisenberg et al. 1981; Shipin et al. 1997; Gutzeit et al. 2005), inhibitors (Toerien et al. 1984), growth-promoting factors (Fukami et al. 1997), antibacterials (Schumacher and Sekoulov 2003), or algicides (Fukami et al. 1997) into the medium. These interactions can have positive, negative, and neutral effects on productivity (Toerien et al. 1984; Munoz and Guieysse 2006). In addition, microbial activity may have negative environmental side-effects such as pH increase or shading, which can reduce bacterial and microalgal growth, respectively. Key interactions that may occur within the microbial flocs are shown in Figure 1-2.

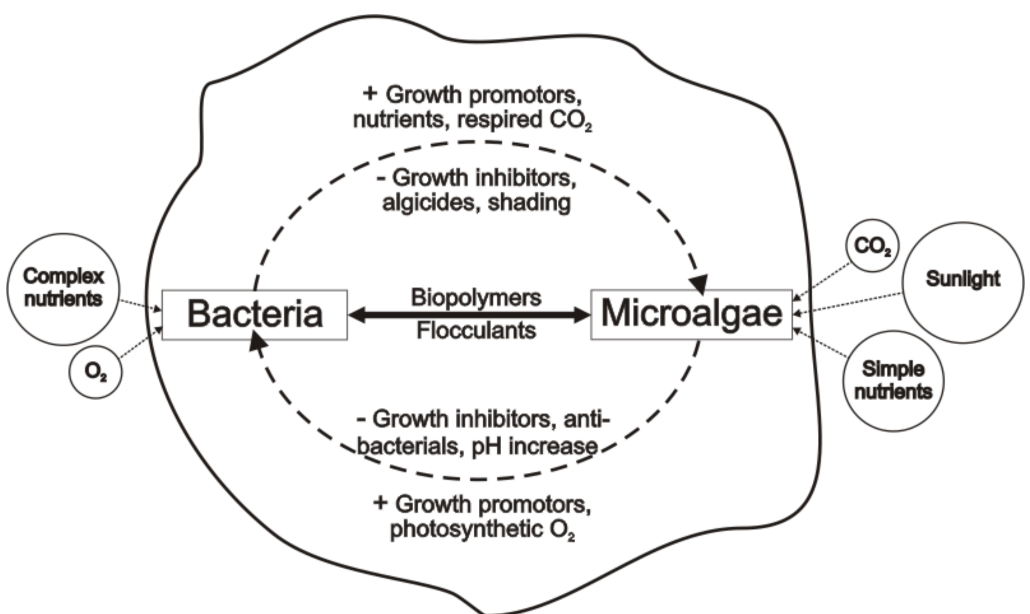

Figure 1-2. Inputs and Symbiosis of a Microbial (Microalgal-Bacterial) Floc.

(Note: positive [+] and negative [-] impacts on growth indicated.)

Microbial flocs may contain at least two-thirds microalgae by mass (Eisenberg 1981; Gutzeit et al. 2005) with the remainder comprised of bacteria and partially treated sewage organics. These flocs have similar characteristics to AS flocs (Medina and Neis 2006), which contain mostly bacteria with filamentous types providing the backbone (Urbain et al. 1993). Additional constituents include inorganic compounds, divalent cations, and extracellular polymeric substances (EPS), which are formed from metabolites, dead microbial cells, and wastewater solids (Urbain et al. 1993) and can affect floc strength and settleability.

Stability and settleability of microbial flocs are paramount when considering biomass harvestability (Section 1.4.6.2). They can be affected by many factors as summarised in Table 1-2. Researchers have not always agreed on how the factors are related. At a basic level, Nielson et al. (2004) state that large, compact flocs are more settleable than small, porous flocs in accordance with fluid dynamics (e.g., Stokes' Law), and strong flocs are easier to dewater resulting in reduced energy demands and smaller handling volumes. Therefore, encouraging formation of stable, large, and compact microbial flocs using specific operational strategies could enhance biomass settleability and harvesting compared to solitary microbes.

Table 1-2. Factors Linked to Stability and Settleability of Microbial Flocs.

Parameter	Reference(s)
EPS content and arrangement	(Urbain et al. 1993; Liao et al. 2001; Liao et al. 2002; Jin et al. 2003; de Schryver et al. 2008)
Hydraulic retention time (HRT)	(Gutzeit et al. 2005; Medina and Neis 2007; de Schryver et al. 2008)
Solids retention time (SRT) or food to microorganism (F/M) ratio	(Liao et al. 2001; Liao et al. 2002; Liao et al. 2006; Medina and Neis 2007)
Carbon source	(de Schryver et al. 2008)
Calcium and other divalent ions	(Jin et al. 2003; Medina and Neis 2006; de Schryver et al. 2008)
Filament index	(Urbain et al. 1993; Jin et al. 2003; Medina and Neis 2006)
Mixing	(Eisenberg 1981; de Schryver et al. 2008)
Dissolved oxygen (DO) and temperature	(de Schryver et al. 2008)
Surface charge or zeta potential	(Liao et al. 2001; Jin et al. 2003; de Schryver et al. 2008)
Hydrophobicity	(Urbain et al. 1993; Liao et al. 2001; Jin et al. 2003; Medina and Neis 2006)

1.4.5 Wastewater Treatment

Wastewater contains an abundant supply of the nutrients required by microalgae and bacteria, and so, concurrent microbial growth and wastewater treatment readily occur (McGriff and McKinney 1972; Eisenberg 1981; Toerien et al. 1984; Gutzeit et al. 2005; Munoz and Guieysse 2006; Kumar et al. 2010a). Photoautotrophic microalgae assimilate inorganic nutrients and produce O_2 which supports the growth of aerobic, heterotrophic bacteria. Bacteria degrade complex organic compounds (e.g., carbohydrates, lipids, proteins, and nucleic acids) and facilitate nitrification and denitrification. Microalgal cultures have been grown on a diverse range of wastewaters as summarised in Table 1-3.

Wastewater is a largely under-capitalised resource with a substantial ability to supply otherwise unutilised nutrients to large-scale microbial production systems. For example, in NZ alone, it was estimated that treating all municipal wastewater in HRAPs could produce about 190 Kt/yr of microbial biomass (Heubeck and Craggs 2007). Moreover, regulations for sewage treatment have become increasingly stringent worldwide with secondary and sometimes tertiary

Table 1-3. Wastewaters Utilised for Microalgal Cultivation.

Sector	Wastewater Type	Reference(s)
Municipal	Raw sewage	(Oron et al. 1979); (Travieso et al. 1996); (Shipin et al. 1999)
	Screened and/or settled sewage	(McGriff and McKinney 1972); (Eisenberg et al. 1981); (Garcia et al. 2006); (Kavanagh and Keller 2007)
	Primary treated wastewater	(Lau et al. 1995); (Gutzeit et al. 2005)
	Primary treated sewage / seawater mixture	(Craggs et al. 1997)
	Secondary treated wastewater	(de la Noüe et al. 1984); (Lavoie and de la Noüe 1987); (Sawayama et al. 1992); (Espigares et al. 1996); (Schumacher and Sekoulov 2003); (Sreesai and Pakpain 2007);
	Pretreated sewage from ponding system	(Banat et al. 1990); (Al-Shayji et al. 1994); (Shipin et al. 1999); (Tadesse et al. 2004)
Agricultural	Settled swine wastewater/ sewage mixture	(Travieso et al. 2006)
	Swine wastewater	(Cañizares-Villanueva et al. 1995); (de Godos et al. 2009); (Kumar et al. 2010b)
	Dairy wastewater	(Lincoln et al. 1996)
	Cattle feedlot effluent	(Toerien et al. 1984)
	Pretreated cattle manure	(Travieso et al. 1996); (Wilkie and Mulbry 2002)
	Aquaculture	(Hammouda et al. 1995); (Brune et al. 2003); (de Schryver et al. 2008)
Industrial	Parboiled rice effluent	(Zepka et al. 2008)
	Carpet mill effluent / sewage mixture	(Chinnasamy et al. 2010)
	Olive oil mill effluent	(Sánchez Villasclaras et al. 1996); (Pinto et al. 2003); (Hodaifa et al. 2008)
	Paper industry effluent	(Tarlan et al. 2002)
	Steel making facility effluent	(Yun et al. 1997)
	Textile effluent	(Lim et al. 2010)
	Tannery effluent	(Ranjitha and Veziroglu 1984); (Rose et al. 1996); (Tadesse et al. 2004)
	Hazardous wastes	(Munoz and Guieysse 2006)

wastewater treatment processes being compulsory for developed countries (Mara and Horan 2003). Additional treatment increases nutrient recovery and reduces environmental impacts, but it also usually incurs significant cost. However, microalgal-bacterial systems can provide a high degree of wastewater treatment while also reducing or even eliminating mechanical aeration requirements. Eisenberg et al. (1981) estimated energy savings of more than 50% for such a system compared to a conventional WTP. Energy requirements of WTPs can be wholly provided from anaerobic digestion of biosolids to generate electricity (Murphy and McKeogh 2006), so this could result in even greater surplus energy being exported to the grid for microbial systems containing microalgae.

1.4.6 Factors Affecting Large-Scale Microbial Biofuel Production

1.4.6.1 Biomass Productivity in Photobioreactors and High-Rate Algal Ponds

Closed photobioreactors (PBRs) and open HRAPs are used for the full-scale production of microalgae (Figure 1-3). Both growth systems offer various advantages and disadvantages (Borowitzka 1999; Lee 2001). PBRs are highly engineered systems that provide an environment in which unialgal cultures can be grown free from contamination or competition by other microbes. They can be flat plates or tubes with horizontal, vertical, inclined, or spiral arrangement (Richmond 2004). A shorter optical path generally makes PBRs more productive than HRAPs (Munoz and Guieysse 2006; Chisti 2007). PBRs can attain volumetric and areal productivities approximately 10- and 30-fold greater than HRAPs (Chisti 2007). Despite their greater yields, some critics have argued that PBRs cannot be economically viable for biofuel production due to their high capital, operating, and maintenance costs (Benemann 2008). Also, according to the laws of thermodynamics, Dimitrov (2007) explained that light conversion efficiency of microalgae (which is usually < 10%) would need to exceed the maximum theoretical limit (i.e., 27%) to justify the economic cost of current PBR designs.

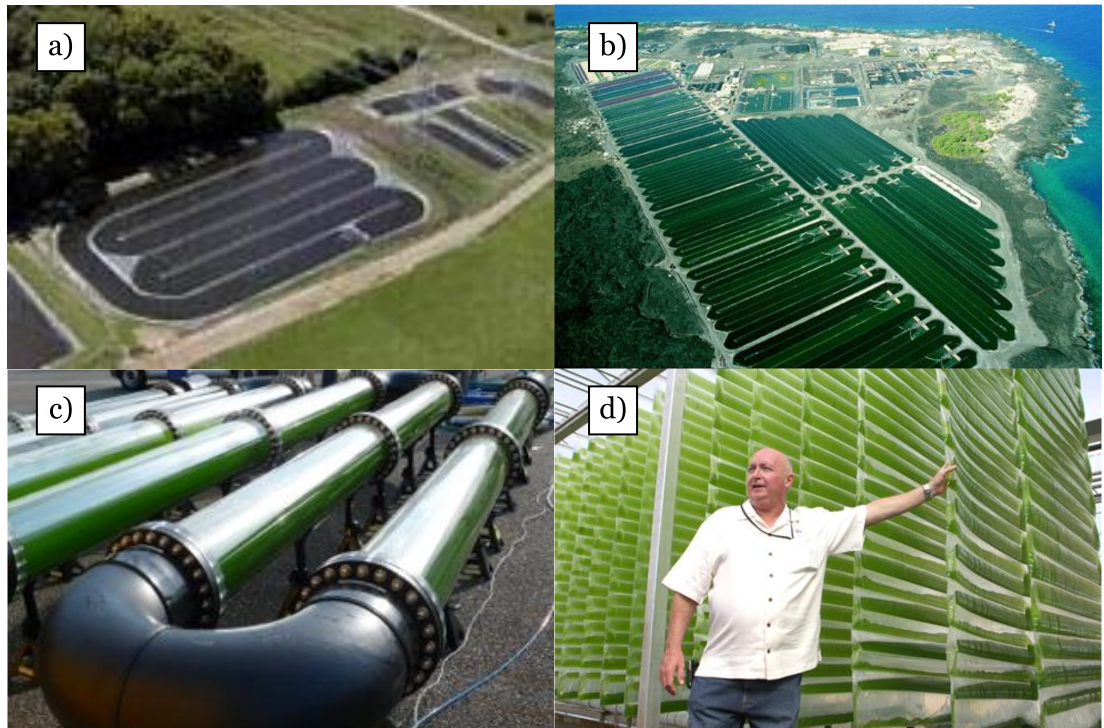

Figure 1-3. Full-Scale Microalgal Growth Systems: HRAPs in a) Hamilton, NZ (NIWA 2007) **and b) Kailua-Kona, Hawaii, USA** (Cyanotech 2007) **and PBRs in c) the Netherlands** (AlgaeLink 2007) **and d) El Paso, Texas, USA** (Valcent Products Inc. 2008)**.**

HRAPs are shallow, raceway-shaped, open ponds (Figure 1-3). They are usually 2-3 m wide, < 0.5 m deep to allow sufficient light penetration, and have a rotating paddlewheel to continuously mix the culture for uniform light exposure. They are termed "high-rate" because of their improved performance (i.e., concurrent biomass productivity and wastewater treatment capacity) compared to deeper, unmixed, open ponds such as oxidation ponds. For example, outdoor, large-scale HRAPs may achieve productivities up to 25 $g/m^2/d$ whereas unmixed ponds rarely exceed 1 $g/m^2/d$ (Richmond 2004). Due to higher durability, reduced maintenance, and other economical considerations, HRAPs are typically favoured over unmixed ponds and PBRs for large-scale cultivation where applicable (Becker 1994; Richmond 2004; Benemann 2008), and they are further supported by LCAs for biofuel production (Jorquera et al. 2010).

Many factors influence HRAP productivity including climate, nutrients, and microbes (Figure 1-4). Temperature and irradiance are directly related to photosynthesis and affect photoinhibition (Richmond 2004; Moheimani and Borowitzka 2007). Low temperatures decrease biological activity and may reduce the threshold of photoinhibition (Richmond 2004). For these reasons, locations with a minimum mean temperature of 15 °C (i.e., between 37 °N and S latitude) are generally recommended for microalgal growth (Van Harmelen and Oonk 2006). Even so, Grönlund (2004) was able to grow cold-adapted microalgae on wastewater in Sweden (i.e., 60 °N) at temperatures below 10 °C. Irradiance limits yield only when nutrients are supplied in excess and temperature is optimal (Becker 1994; Richmond 2004). Indirect effects of microalgal activity such as elevated pH and dissolved oxygen (DO) from photosynthesis can also reduce productivity—pH > 9 and 10 standard pH units (SU) can inhibit

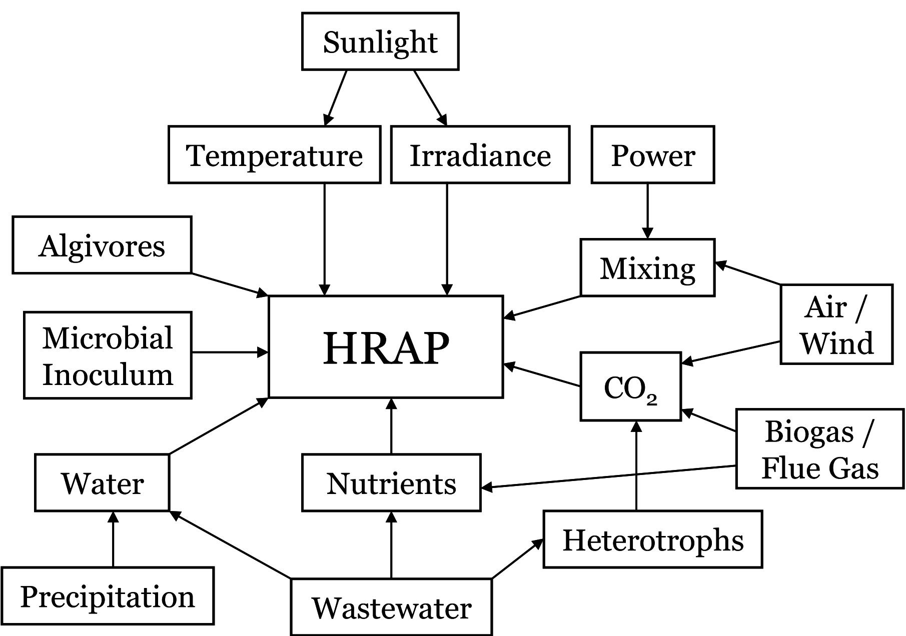

Figure 1-4. Biotic and Abiotic Factors Affecting Microalgal Productivity in HRAPs.

bacterial and microalgal growth (Jimenez et al. 2003; Mara and Horan 2003), respectively, and supersaturated DO can damage microalgal cells (Marquez et al. 1995; Jimenez et al. 2003; Richmond 2004). Proper mixing is also important for reducing self-shading of microalgal cells, facilitating light/dark cycling, and promoting homogenous growth conditions (Becker 1994; Mihalyfalvy et al. 1998), which thereby produces up to 25 times greater biomass than unmixed cultures (Richmond 2004). Since paddlewheels reduce shear stress and are cost-efficient, they are often preferred for pond mixing by generating velocities of 15 to 25 cm/s (Benemann and Oswald 1996; Munoz and Guieysse 2006). Lastly, algivores and other contaminants may need to be managed to maintain healthy biomass yields (Becker 1994). For example, uncontrolled ciliate populations can consume up to 41% of microalgal biomass in one day (Graham et al. 2009).

Many researchers have attempted to determine optimal conditions for microalgal growth in outdoor systems (e.g., Cromar et al. 1996; Jimenez et al. 2003; Moheimani and Borowitzka 2007). Unfortunately, relationships between controlled operational parameters and resulting biological responses are often complex and confounding due to the inherent variability and diversity of living systems and the different climates under which they are studied. Variables such as nutrient supply can be easily adjusted in feed water, but the outdoor environment is changeable and uncontrollable. Moreover, significant physiological differences exist between microalgal species and may even evolve among different strains of the same species due to environmental adaptation (Sheehan et al. 1998). Because these differences can have substantial impacts on microalgal productivity, site-specific studies are necessary to thoroughly assess microbial response to prevailing conditions before implementing full-scale biotechnologies.

1.4.6.2 Biomass Harvesting

Effective separation of microalgae from their growth medium is challenging due to their dilute growth concentrations, physiology, and density (Becker 1994). Harvesting contributes 20-30% to total microalgal production cost (Gudin and Thepenier 1986) by concentrating biomass from 50- to 200-fold using centrifugation, filtration, flocculation with sedimentation, or flocculation with flotation. Suitability of the selected process depends on the microalgal morphology, desired end-products, and projected cost. Commonly utilised primary harvesting methods are summarised in Table 1-4 and discussed briefly below.

Centrifugation: Industrial centrifuges concentrate microalgae in large, quickly rotating chambers achieving forces of several thousand times gravity. They are very effective, but they have significant capital and operating costs (Benemann and Oswald 1996). The dilute concentration and large volumes of microalgal cultures preclude this method as a primary harvesting step.

Table 1-4. Comparison of Microalgal Biomass Harvesting Methods.

Method	Major Inputs	Energy Input	Relative Cost	Dependence on Species Morphology	Solids Concentration Capability
Centrifugation[a]	Power, equipment	High	10	Low	> 10%
Pressure filtration[b]	Power, equipment	Medium	4-5	High	5-27%
Vacuum filtration[b]	Power, equipment	Medium/ High	9-40	High	9.5-37%
Microstraining[a]	Power, equipment	Medium	0.5-1.5	High	2-4%
Chemical flocculation[a]	Chemicals, power	High	6-8	Low	8-10%
Bioflocculation[a]	Power, equipment	Low	0.5-1.0	High	1-3%

Note: values approximated for comparison purposes only; costs shown relative to that of centrifugation on an arbitrary scale; (a) adapted from Benemann and Oswald (1996); (b) adapted from Richmond (2004).

Filtration: Pressure (e.g., chamber) and vacuum (e.g., drum and belt) filters (Molina Grima et al. 2003) and microstrainers (Benemann and Oswald 1996; Uduman et al. 2010) are satisfactory for solid/liquid separation. However, biomass recovery by these devices decreases as microalgal cell size decreases. Unless the microbial community primarily consists of large (i.e., > 50 µm), colonial, and/or filamentous microalgae, filter clogging and cell breakthrough commonly occur (Mohn 1980).

Chemical flocculation: Flocculants are generally classified as inorganic compounds (e.g., aluminium and iron salts), organic synthetic high-polymer compounds (e.g., polyethyleneamine), or naturally occurring compounds (e.g., chitosan). These substances neutralise, bridge, bind, and flocculate microalgal cells in water and wastewater treatment processes which enable them to settle faster than free-floating microalgae due to a higher specific gravity and, thus, settling velocity. Flocculation is more convenient than centrifugation or filtration because it allows large volumes of water to be processed quickly. However, flocculants are an added expense, and they can limit use of the harvested biomass for biofuels, animal feed, and fertiliser applications due to unwanted chemicals.

Natural floc formation: Microalgae and bacteria can combine naturally into readily settleable flocs. Natural floc formation occurs through nutrient limitation, CO_2 limitation, elevated pH, and water hardness (i.e., Ca^{2+} and Mg^{2+} levels) (Becker 1994). For example, it can be induced by extending HRT, which reduces nutrients available for microbial growth. Under long HRT conditions, however, productivity can be significantly reduced, so improved harvesting efficiency must offset lower yields. Floc formation can occur spontaneously as a result of bioflocculation when mixing ceases by allowing microbial aggregation and natural sedimentation. Biotic factors such as growth stage (i.e., cell age) and microalgal-bacterial

interactions outlined earlier may also impact bioflocculation. For instance, microalgae have a high negative surface charge during exponential growth, which keeps cells separated by repulsive forces, but when the charge reduces during periods of low growth, bioflocculation has been observed (Becker 1994; Henderson et al. 2008).

Natural floc formation can also occur gradually as microbes aggregate into stable, compact flocs. Within this thesis, stable, compact flocs are defined as those ones that do not dissociate under normal system operating conditions. Many microorganisms, such as those found in AS, naturally excrete EBFs into their culture causing stable flocs to form. EBF production can be influenced by physiological and environmental factors including carbon to nitrogen (C/N) ratio, metal ions, pH, temperature, and mixing speed (Salehizadeh and Shojaosadati 2001). Li et al. (2007) found that EBFs were produced by AS only during stationary growth, which may indicate a preferential harvesting period. Cations (e.g., Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, and Al^{3+}) in the culture may also encourage floc formation and improve settleability by: 1) bridging microbes and EBFs (Sobeck and Higgins 2002; Jin et al. 2003; Chen and Yeh 2005; Li et al. 2007) and 2) neutralising the negative surface charge of the flocs (Becker 1994; Jin et al. 2003; Chen and Yeh 2005). Perhaps due to all of these factors, good biomass settleability has been observed for mixed *Chlorella*-AS cultures (Humenik and Hanna 1971; Aziz and Ng 1992; Gutzeit et al. 2005) probably due to the role of AS as a flocculating agent in mixed microalgal cultures. Overall, the mechanics of natural microalgae sedimentation are complex and variable yet remain an important challenge to be better understood and optimised for sustainable biomass production.

Following floc formation, the microbial biomass can be concentrated by flotation or gravity sedimentation. Air flotation is an energy-intensive process that injects tiny bubbles of air (10-100 µm) into the growth medium to lift solids to the surface for removal by skimming or other methods (Uduman et al. 2010). Sedimentation enables decantation of clear supernatant after a quiescent settling period, which effectively dewaters the settled solids. Because natural floc formation followed by sedimentation is the most sustainable biomass harvesting approach (i.e., requiring the lowest energy, chemicals, and capital investment), it was investigated as part of this research. This method was recommended by the ASP (Sheehan et al. 1998) for large-scale biofuel applications and also supported in a feasibility report for Christchurch, NZ by Beca Infrastructure Ltd. (2007).

1.4.6.3 Biochemical Composition

The composition of biomass can significantly impact its quality for biofuel purposes. Microbial biomass principally consists of lipids, proteins, and carbohydrates. Small quantities of minerals, nucleic acids, sterols, pigments, and vitamins are also present. The relative proportions of these constituents can be highly affected by environmental conditions. For example, in microalgae, N limitation can stimulate lipid accumulation (Benemann and Oswald 1996; Sheehan et al. 1998),

low water temperatures can enhance polyunsaturated fatty acid (PUFA) production (Becker 1994; Blanchemain and Grizeau 1999), and high light intensities can increase carbohydrate synthesis (Richmond 2004). Manipulating biomass concentration can also be an effective way to alter the biochemical composition of microalgae (Hu et al. 1997; Richmond 2004) since this variable impacts availability of light and nutrients. Increasing solids recycling, for instance, could increase TSS while causing N limitation to increase lipid content. Inducing unfavourable temperature or nutrient deprivation to alter composition, however, will reduce biomass productivity and could cause culture instability and collapse.

The desired biofuel determines the relative importance of the chemical properties of the biomass produced. Lipid content and type influence the quantity and quality of biodiesel produced from transesterification. Highly saturated fatty acids (SFAs) are desired for conversion into biodiesel as they have greater storage stability and are less likely to polymerize during combustion (Sheehan et al. 1998). Meanwhile, C/N ratio influences biogas production from anaerobic digestion. Generally, a C/N ratio of 20-30 is recommended for optimal CH_4 yields (Ward et al. 2008).

1.4.6.4 Economics

Large-scale microalgal biofuel production has yet to be demonstrated. Currently, commercial production is generally less than a few hundred tonnes annually per supplier and targeted for human nutrition through *Spirulina* and similar products (Richmond 2004; Benemann 2008). Many researchers have cautioned that stand-alone biofuel production from microalgae or other feedstocks will not be economical (Benemann et al. 1982; Sheehan et al. 1998; Benemann et al. 2003a; Moheimani 2005; Van Harmelen and Oonk 2006; Chisti 2007). However, coupling microbial biofuel production to other processes such as wastewater treatment, CO_2 sequestration, and/or fertiliser production capitalises on nutrient recycling pathways and could improve its economic and environmental outlook.

Economic (and political) changes clearly impact prices of fossil fuels, equipment, labour, revenues, and other factors related to the practicality of large-scale microbial biofuel production systems. Several feasibility studies have been conducted for HRAPs over the years based on various details and assumptions (Benemann et al. 1982; Weissman and Goebel 1987; Anderson 2002; Beca Infrastructure Ltd. 2007). Benemann and Oswald (1996) reviewed previous analyses and determined that updated operating and capital costs ranged from $63-284/barrel (i.e., adjusted from 1994 to 2010 $US) depending on productivity and capital expenditure, which largely exceeds today's crude oil price of approximately US$77/barrel (Energy Information Administration 2010). However, revenues realised from biofuel sales, reclaimed water, CO_2 abatement, and co-products (e.g., fertilisers, animal feed, polymers) could substantially offset the overall cost to make biofuels a more economically attractive alternative to petroleum.

Assuming 30% oil in the biomass, accrued revenues of $95-570/t equating to $43-260/barrel (i.e., adjusted from 2006 € to 2010 $US) (Van Harmelen and Oonk 2006) could make microbial biofuel production economical. The wide price ranges are highly dependent on 1) feedstock production cost (i.e., based on growth system, cultivation techniques, and biomass yield), 2) biofuel conversion process, 3) revenues, and 4) economic conditions. To reduce these uncertainties, site-specific economic assessments based on demonstration studies should be prepared to ascertain feasibility of any proposed large-scale biofuel production facility.

1.5 Research Drivers

Further research can improve microalgal biofuel technology by addressing the challenges of high biomass productivity and harvesting efficiency. Today, people are also starting to recognise the importance of sustainable, integrated systems–ones that meet environmental, societal, and economical needs. Hence, an ecological engineering approach considering natural energies, conservation, and self-design of the ecological communities (Mitsch and Jorgensen 2003) was used to better meet these challenges. These key principles of ecological engineering were satisfied by (1) inoculating systems with native microbes and relying on natural selection to form stable communities, (2) treating wastewater by recycling waste nutrients, and (3) using natural sedimentation to reduce non-renewable energy usage.

Current unknowns surrounding microalgal-bacterial biomass production are the effects of wastewater, climate, and retention time on biomass productivity, settleability, and composition. While conditions of wastewater, climate, and ecology are site-specific, their combined influence is inherent and relevant to open, practical systems, so these factors should not be studied in isolation when relying on an ecosystem approach. Various feed waters have been used for microalgal and/or bacterial growth, but they have not been compared to each other under the same biological, environmental, and operational conditions. Moreover, physiological differences among microbial communities can have significant impacts on growth. There has also been little research into retention time impacts and settleability responses. This research can contribute new knowledge to feasibility assessments aimed at large-scale microbial biofuel applications.

1.6 Research Hypotheses, Objectives, and Organisation

This research sought to investigate sustainable microbial (microalgal-bacterial) biomass production. It was hypothesised that biomass productivity, settleability, and composition would be impacted by wastewater, climate, and retention time, and that conditions could be sequentially optimised to improve yield. The optimisation strategy and specific research objectives are presented in Figure 1-5. While wastewater treatment occurred as a consequence of the microbial growth, meeting water quality discharge requirements was considered a bonus rather than a goal of this research.

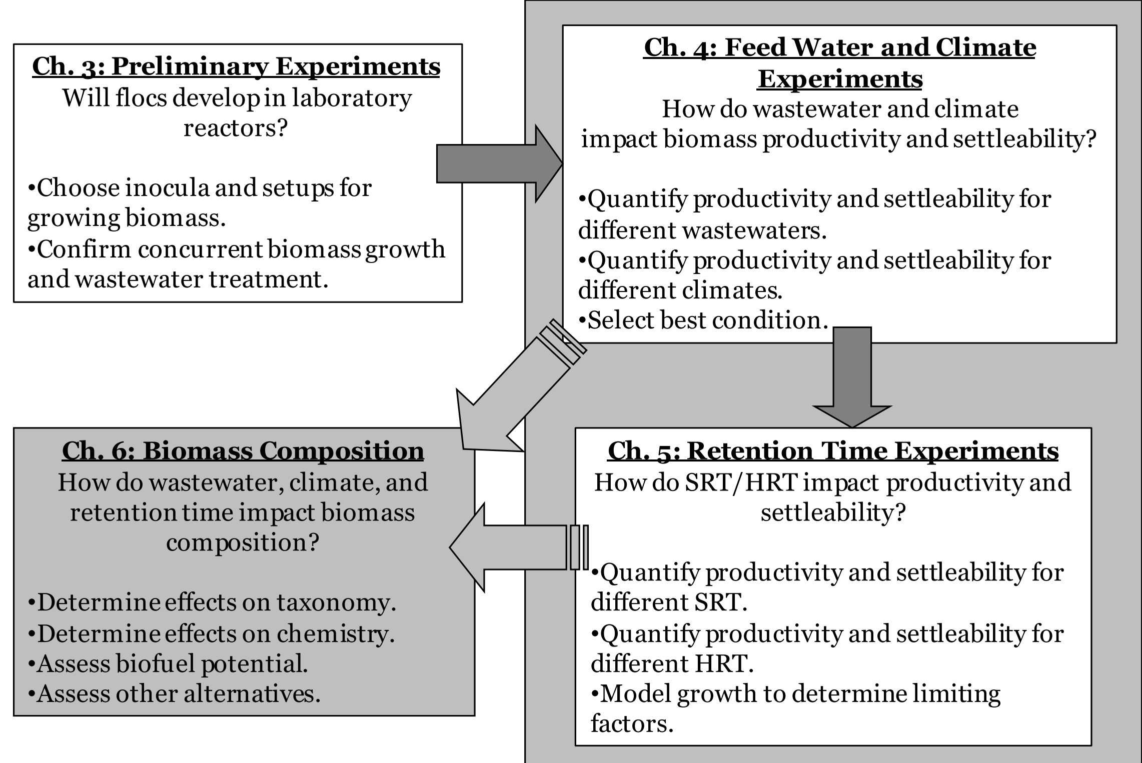

Figure 1-5. Research Objectives and Thesis Progression.

Using defined Materials and Methods (Chapter 2), several experiments were conducted to test the hypotheses and meet these objectives. Initially, inocula and some operational strategies were explored (Chapter 3). Next, feed water condition was selected (Chapter 4), and retention times were investigated while assessing growth limitation (Chapter 5). Implications for use of the resulting biomass based on its composition were then explored using additional data collected during the feed water, climate, and retention time experiments (Chapter 6). Finally, a summary and future research recommendations resulting from this work were provided (Chapter 7).

Chapter 2: Materials and Methods

2.1 *Source Water Characteristics*

The CWTP serves approximately 340,000 people in Christchurch, NZ. Waste sources are approximately 90% municipal sewage and 10% industrial trade wastes. A network of over 90 pumping stations provides the plant with an average flowrate of 171,000 m^3/d. A flow diagram of the wastewater treatment process (Figure 2-1) and an aerial photograph of the oxidation ponds (Figure 2-2) indicate the extraction points for wastewater sampling. Oxidation pond wastewater (from Pond 1 [P1] and Pond 6 [P6]) and activated sludge (AS) were used as microbial inocula, and primary and secondary treated wastewaters were used as feed waters. The combined mixture of any inoculum(s) and the corresponding wastewater feed was generally referred to as a 'culture.' Wastewater characteristics monitored by CWTP as part of its regular monitoring regime are summarised in Table 2-1.

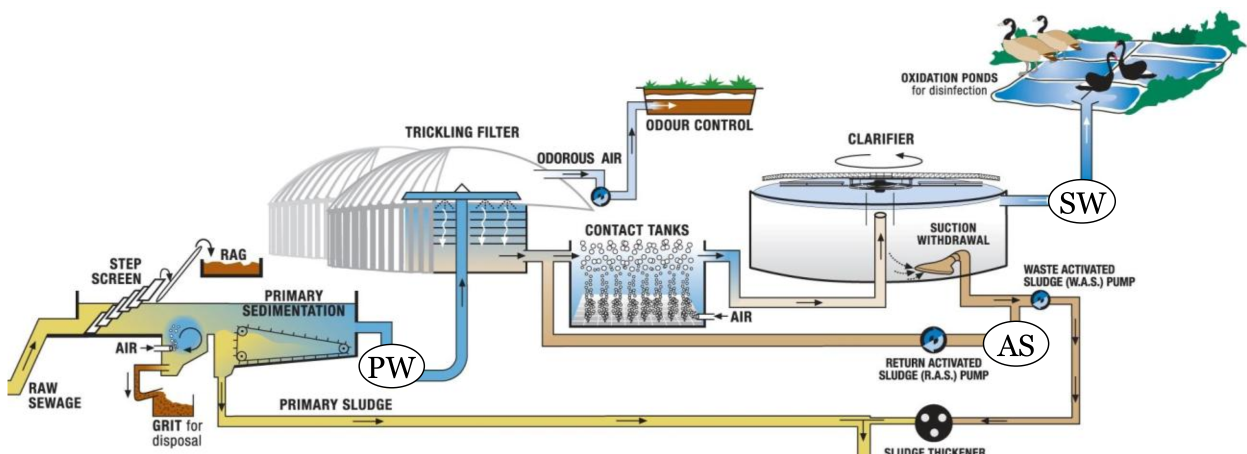

Figure 2-1. Diagram of Municipal Wastewater Treatment Process at CWTP, NZ.
(Note: adapted from Christchurch City Council [2009]; extraction points for primary treated [PW], secondary treated [SW], and activated sludge [AS] wastewaters shown.)

2.1.1 *Microbial Inocula*

Oxidation Pond Water: Oxidation pond wastewater contained an assortment of microbes including indigenous microalgae and bacteria. In addition to native microbes being more likely to survive in laboratory studies conducted under similar growth conditions, strict containment requirements mandated by the Hazardous Substances and New Organisms (HSNO) Act (Packer 2009) were avoided. The microbial communities varied over time (Appendix A) in response to changes in site-specific conditions (e.g., climate, algivores, nutrients, and contaminants) as was also observed by Novis (2007). Seven oxidation ponds (P1, P2a, P2b, P3, P4, P5, and P6) arranged in series (Figure 2-2) facilitated UV disinfection of the wastewater before discharge to Pegasus Bay. (Previously, wastewater was discharged into the Avon-Heathcote Estuary until

Figure 2-2. Aerial Photograph of Oxidation Ponds at CWTP, NZ.

(Note: adapted from Christchurch City Council [2003]; wastewater extraction points for oxidation pond 1 [P1] and pond 6 [P6] shown.)

March 2010.) Characteristics of P1 wastewater data were unavailable, but P1 receives secondary treated wastewater and precedes P6, so its characteristics were expected to be within the range of secondary treated and P6 wastewaters (Table 2-1). Unless otherwise indicated, oxidation pond water was filtered using 80-µm mesh to remove any larger matter. Growth systems (Section 2.2) were inoculated with the filtrate, or inoculum standardisation was initiated (Section 4.1.1) within 6 h of sampling from CWTP.

Activated Sludge: AS consisted predominantly of microbial biomass and adsorbed particulates settled in the clarifier (Figure 2-1). (Any coarse solids were removed upstream as primary sludge.) Characterised in Table 2-1, AS was used as an inoculum to supply additional flocculating microbes to select reactors within 6 h of sampling from CWTP.

2.1.2 Wastewater Feeds

Primary and secondary treated wastewaters (simply referred to as primary and secondary wastewaters henceforth) (Table 2-1) were collected weekly or biweekly at dedicated sampling locations (Figure 2-1). Primary treatment consisted of screening, grit removal, and sedimentation for removal of suspended matter from raw wastewater to generate primary

Table 2-1. CWTP Wastewater Characteristics during Experiments.

Parameter	Unit	Primary Treated Wastewater[(a)] *M* ± *SD*	Secondary Treated Wastewater[(b)] *M* ± *SD*	Oxidation Pond 6 Wastewater[(a)] *M* ± *SD*	Activated Sludge[(a)] *M* ± *SD*
Chl *a*	mg/L	0.0*	NA	0.4*	0.3—0.8*
Total Carbon (TC as C)	mg/L	104—155*	NA	45—56*	1,427—1,515*
Total Organic Carbon (TOC as C)	mg/L	95—127*	NA	19—33*	779—1,312*
Total BOD	mg/L	156 ± 60	18 ± 8.8	13 ± 7.6	NA
Soluble BOD	mg/L	75 ± 31	5.7 ± 3.2	3.3 ± 2.0	NA
Total COD	mg/L	400 ± 125	90 ± 64	92 ± 33	NA
Soluble COD	mg/L	188 ± 57	51 ± 9.2	42 ± 9.2	NA
Total Nitrogen (TN as N)	mg/L	44 ± 9.6	37 ± 8.0	29 ± 6.3	NA
Total Kjeldahl Nitrogen (TKN as N)	mg/L	45 ± 12	32 ± 8.2	26 ± 7.9	460—470*
Ammonia Nitrogen (NH_3 as N)	mg/L	30 ± 9.2	27 ± 6.0	21 ± 8.5	NA
Nitrate Nitrogen (NO_3 as N)	mg/L	0.3 ± 0.7	3.4 ± 1.9	1.6 ± 3.3	NA
Nitrite Nitrogen (NO_2 as N)	mg/L	0.3 ± 0.2	0.5 ± 0.7	1.3 ± 2.2	NA
pH	SU	7.4 ± 0.2	7.4 ± 0.1	8.2 ± 0.6	NA
Total Phosphorus (TP as P)	mg/L	6.5 ± 1.6	5.6 ± 3.3	4.7 ± 1.1	95—98*
Soluble Phosphorus (PO_4 as P)	mg/L	4.7 ± 1.1	4.3 ± 0.9	3.8 ± 1.1	NA
Temperature	°C	16.9 ± 2.7	17.7 ± 2.2	15.1 ± 8.4	NA
Total Suspended Solids (TSS)	mg/L	144 ± 112	33 ± 17	36 ± 22	4,007 ± 805
Volatile Suspended Solids (VSS)	mg/L	115 ± 50	20 ± 14	32 ± 21	3,383 ± 731

Note: mean (*M*) ± standard deviation (*SD*) of data provided by CWTP for routine process samples from (a) 01/02/08 to 30/04/10 and (b) 01/07/08 to 30/04/09; range (*) collected by researcher (*n*=2); NA = not available.

wastewater. Next, trickling filters followed by aeration in contact tanks enabled biological degradation of primary wastewater contaminants (secondary treatment). The resulting microbial (activated) sludge was settled in clarifiers to generate secondary wastewater. Primary wastewater was comparable, but secondary wastewater was slightly stronger than that generalised by others (Tchobanoglous et al. 2003). City supply water (i.e., natural and without chlorine, fluoride, etc.), was collected from a tap at University of Canterbury (UC). Unavoidably, some degradation occurred (Appendix B) while feed waters were stored at approximately 4 °C until used during the experiments.

2.2 Laboratory Growth System Specifications

2.2.1 Glass Reactors

Laboratory glassware was used for initial experimental setups (Figure 2-3). It was cleaned with mild detergent (Dri-Decon) and rinsed two times with deionised (DI) water before use. The

glassware's contents were mixed continuously using a magnetic stirrer with an adjustable speed (VELP Scientifica model AGE) and a stir bar (or flea). Mixing was estimated at < 100 rpm. Incandescent light bulbs (Philips 400-W) provided illumination for 12 h/d. Experiments were conducted using these reactors as further detailed in Table 2-2.

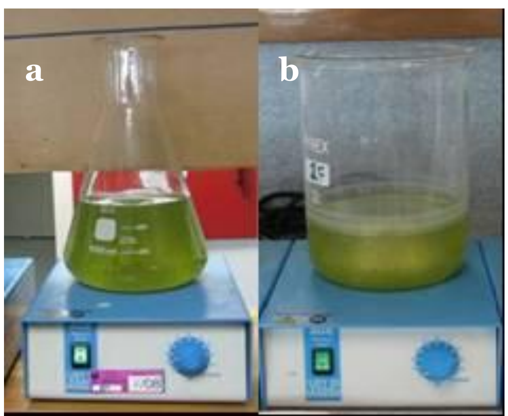

Figure 2-3. Glass Laboratory Reactor Setups using Magnetic Stirrers for a) 1-L Erlenmeyer Flask and b) 2-L Beaker.

2.2.2 Steel Reactors

The reactors consisted of an inlet, a stainless steel tank, a mixer, an incandescent light source, an outlet, and associated valves and piping (Figure 2-4). A data acquisition and control box provided an interface between monitoring instruments and a computer for continuous data logging, and it controlled (on/off) auxiliary equipment (e.g., lights, mixers, pumps) using LabVIEW software (National Instruments 2007). This feature enabled automatic operation of some experiments.

Before initial use, reactors were cleaned with mild detergent, sterilised with bleach, and thoroughly rinsed with hot tap water. Between subsequent experiments, they were cleaned with hot tap water containing mild detergent and rinsed twice with tap water. The major components are described below.

Table 2-2. Operational and Climatic Conditions of Laboratory Growth Systems.

Parameter / Thesis Section	Chapter 3						Chapter 4		Chapter 5
	3.1.1	3.1.2.1	3.1.2.2	3.2	3.3	3.3.4	4.1	4.1	5.1
Culture Abbreviations	C1, C6, C6F	0, 5, 10% AS	C1-C7	R1, R2	SBR1, SBR2	D50, D75	CO, SE, PE, AP	CO, SE, PE, AP	S4, S8, S12
Replicates (*n*)	1	1	1	2	2	2	2	3	≤ 2
Operational Conditions	*Batch*	*Batch*	*Jar Test*	*Continuous*	*Fed-batch*	*Batch*	*Fed-batch*	*Fed-batch*	*Fed-batch*
Reactor Material	Glass	Glass	Glass	Steel	Steel	Glass	Steel	Steel	Steel
Reactor Volume (L)	1.15	0.8	1.0-1.3	25	21	2	21-24	21-24	22.5
Reactor Depth (cm)	10	9	9-11	35	30	17	30-34	30-34	32.5
Mixing Speed (rpm)	< 100	< 100	25	50	50	< 100	50	50	50
Illuminated Surface (cm^2)	120	-	120	707	707	120	707	707	707
Reaction Phase (d)	8	14	-	-	3	12	1	1	1
Hydraulic Retention Time (d)	-	-	-	7	9	-	8	8	4, 2, 1.4
Solids Retention Time (d)	-	-	-	7+	9-80	-	8-40	8-40	4, 8, 12
*Climatic Conditions**	*Ambient*	*Ambient*	*Ambient*	*Ambient*	*Ambient*	*Ambient*	*Cold*	*Warm*	*Warm*
Daytime PAR (μmol/m^2/s)	NA	NA	NA	390	516	516	410	925	925
Day Length (h)	12	12	-	12	12	12	9.6	14.7	14.7
Water Temperature (°C)	33	NA	24	21 ± 1	25 ± 3	25 ± 3	13 ± 3	21 ± 3	19 ± 3

+Some unintentional biomass recycling occurred. *Climate conditions further detailed in Section 2.6. NA = not available.

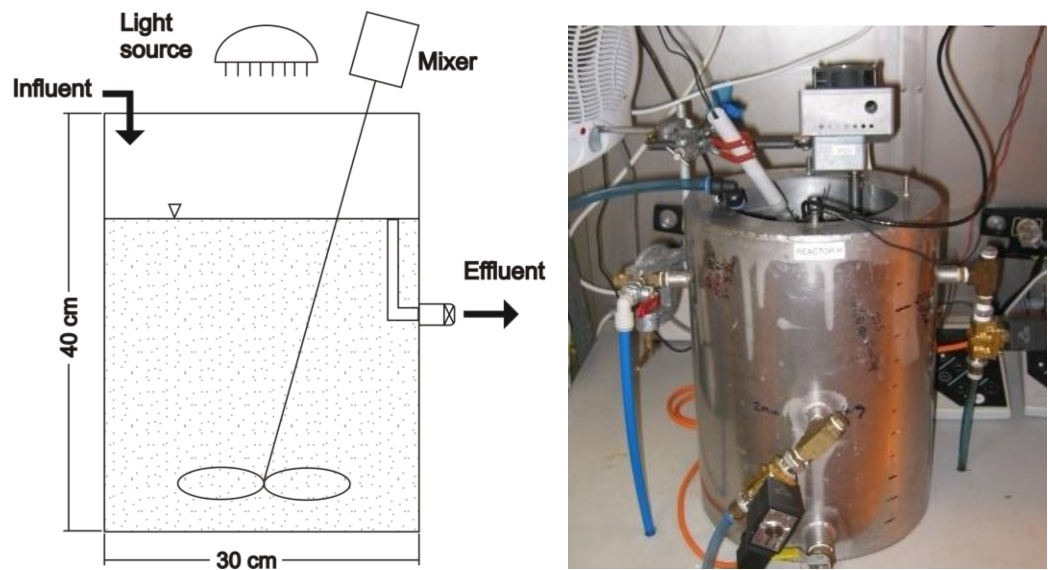

Figure 2-4. Schematic (Left) and Photograph (Right) of Steel Reactor Setup.

- **Influent.** Feed wastewater (Section 2.1.2) was premixed manually or automatically using a submersible pump (Little Giant 5-MSPR). It was supplied to reactors manually or via a peristaltic (Masterflex) or a submersible pump (Little Giant, 2E-38N) to attain hydraulic residence times (HRTs) ranging from 1.4-9 d. Reactors were either drip-fed continuously or fed only at the beginning of each reaction phase (RP). Dedicated pump calibration and/or the use of float switches ensured specific HRTs were maintained.
- **Growth Tank.** Laboratory reactors had a 0.3-m diameter and 0.4-m height for a maximum volume of 28 L. A mixer (RS Type 718-852 or Cole Palmer Masterflex pump head) operating at 50 rpm and outfitted with a 10-cm × 7.3-cm stainless steel blade provided a flow velocity of 26 cm/s, which was within the 15-30 cm/s range recommended by Sheehan et al. (1998) to promote flocculation. Incandescent light bulbs (Philips 400-W) illuminated the 707 cm^2 surface of cultures for preset time periods to achieve diurnal light/dark periods. The height of light bulbs above the water surface was varied as needed to control photosynthetically active radiation (PAR). Air fans were used to dissipate some of the heat load from the bulbs to the cultures during light periods.
- **Monitoring.** Water temperature, pH, DO, and PAR were measured at 15-min intervals using various sensors (Section 2.4.1). Other analytes (Section 2.4) were monitored less frequently to meet research objectives.
- **Effluent.** Discharge ports and sample taps at heights of 5, 20, and 30 cm enabled sample collection and water depth control. An adjustable standpipe and/or automated valve connected to a sampling port were used to obtain other depths and operating volumes via overflow. Reactors were discharged continuously or at the end

of each RP. Effluents consisted of wasted supernatant (i.e., unsettleable material including microbes) and/or mixture.

Reactors were operated as continuous stirred tank reactors (CSTRs) or sequencing batch reactors (SBRs) with and without solids recycling. CSTRs were fed, mixed, and discharged continuously (i.e., no settle or supernatant decant phases) while undergoing light/dark periods. SBRs proceeded through feed, mix, settle, and waste steps according to Figure 2-5. This sequence recycled settleable solids and, thus, selected for the growth and formation of compact microbial flocs rather than dispersed microbes. Operating specifications are provided in Table 2-2 and further detailed for each fed-batch (SBR) sequence (Appendix B). Different ratios of mixture and supernatant were removed following 60-min sedimentation periods to obtain specific HRTs and solids retention times (SRTs). HRTs and SRTs were calculated according to Equations 2-1 and 2-2.

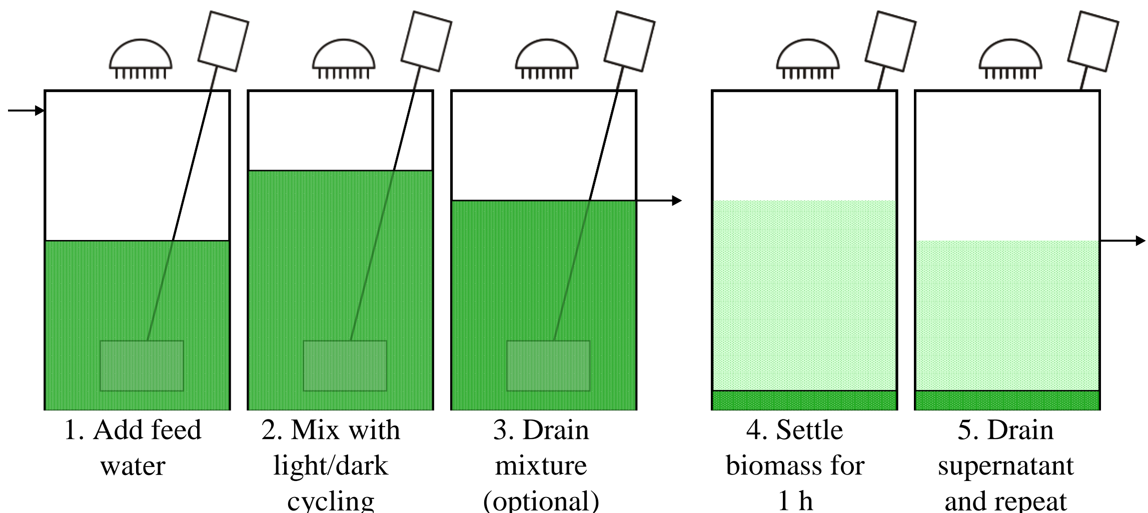

Figure 2-5. Operating Sequence of Laboratory SBRs for One Reaction Phase.

$$HRT = \frac{V_r}{Q_m + Q_s} \quad (2\text{-}1)$$

$$SRT = \frac{V_r}{[Q_m + Q_s(1 - S/100)]} \quad (2\text{-}2)$$

Where: *HRT = hydraulic retention time (d)*
V_r = reactor volume (L)
Q_m = mixture discharge flowrate (L/d)
Q_s = supernatant discharge flowrate (L/d)
SRT = solids retention time (d)
S = settleability (%) (Section 2.4.3)

2.3 *Sampling Logistics*

2.3.1 *Mixture*

Mixture (grab) samples were collected via a sample tap or from the reactor's surface while the contents were completely mixed (i.e., Figure 2-5, Step 2). Dry weight (dw) analysis of samples collected at the top and bottom of the water column confirmed that reactors were completely mixed (Appendix B).

2.3.2 *Supernatant*

Supernatant samples were collected from the decanted effluent (Figure 2-5, Step 5) or an Imhoff Cone after the mixture was allowed to settle in darkness without mixing for 60 min. Imhoff Cone samples were collected from halfway between the solid-liquid interface and the water surface (per standard method [SM] 2540F; American Public Health Association [APHA] 2005) (Figure 2-6, Step 2). Actual sampling depth within the cone depended on the volume of settled solids.

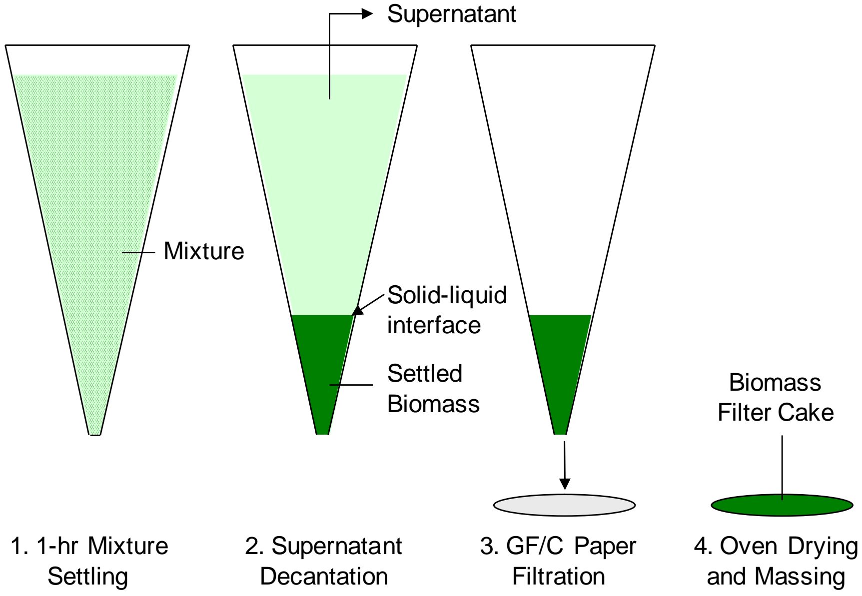

Figure 2-6. Imhoff Cone Sample Processing for Supernatant and Settleable Biomass.

2.3.3 *Settleable Biomass*

A new method was developed to quantify settleable biomass. After 1 L of mixture had settled for 60 min in an Imhoff Cone (in accordance with SM 2540F; APHA 2005) (Figure 2-6, Step 1), its supernatant was decanted to the solid-liquid interface (Figure 2-6, Step 2). Settled biomass was then filtered through glass microfiber filter paper (Whatman GF/C) (Figure 2-6, Step 3) to obtain a filter cake of biomass (Figure 2-6, Step 4).

2.3.4 *Total Biomass*

At the end of experiments, supernatant was decanted, and the remaining volume was settled overnight. In the morning, supernatant was again decanted to the new solid-liquid interface to concentrate solids into graduated glassware and/or an Imhoff Cone, and settled volume (SV) was determined. The resulting sludge was dried in an incubator (Contherm Digital Series) at 60 °C (e.g., for subsequent lipids analysis [Section 2.4.8]) or at 105 °C for 12-72 h depending on sample size and temperature.

2.4 *Analytical Methods*

2.4.1 *pH, DO, Water Temperature, and PAR*

At 15-min intervals, the pH, DO, water temperature, and PAR were logged using data loggers connected to computers operating LabVIEW software (National Instruments 2007). The pH was measured using EDT pH Meters (Series 3), and probes were calibrated with fresh 4.0, 7.0, and 10.0 SU buffers. DO was measured using YSI Model 57 Oxygen Meters, and probes were calibrated to supersaturated oxygen levels using air-sparged reverse osmosis water. DO saturation was calculated according to Equation 2-3 (Wang et al. 1978). Weekly cleaning and recalibration of these probes minimised drift. Water temperature was measured using Precision Centigrade Temperature Sensors (LM35) and confirmed weekly using an Ebro TFX 410 handheld thermometer.

$$DO_{sat} = 100 \times \frac{DO_{con}}{(14.61996 - 0.40420T + 0.00842T^2 - 0.00009T^3)} \qquad 2\text{-}3$$

Where: *DO_{sat} = dissolved oxygen saturation (%)*
DO_{con} = dissolved oxygen concentration (mg/L)
T = water temperature (°C)

PAR was measured at the water surface by Apogee Quantum Sensors (model QSO-S). The sensors were factory calibrated for sunlight, but no data manipulation was required (per the manual) for the metal halide lamps used in the laboratory studies. The sensors were cleaned weekly to ensure that no dust or debris accumulated on the lenses. PAR output by the lamps according to the data logger was confirmed weekly using another Apogee Quantum Sensor amplified with an EDT meter.

2.4.2 *Biomass and Dry Weight*

Total solids (TS) (SM 2540B), total suspended solids (TSS) (SM 2540D), volatile solids (VS) (SM 2540E), and volatile suspended solids (VSS) (SM 2540E) were measured according to APHA (2005) using a Contherm Thermotec 2000 oven and a Labec CEMLL-SD or McGregor muffle furnace (for volatiles). TSS analyses estimated microbial biomass concentration (Becker 1994). Chemical oxygen demand (COD), VS, and/or VSS analyses were also conducted in

tandem to quantify the carbonaceous and organic contents. Biomass production of long-term, stable microbial cultures was quantified as areal or volumetric productivity in agreement with the scientific community (Equations 2-4a and 2-4b). Short-term cultures or those not operated under stable conditions were quantified simply using TSS.

$$P_a = \frac{Q_m \times X_m + Q_s \times X_s}{A_s/10} \quad \text{2-4a}$$

$$P_v = \frac{Q_m \times X_m + Q_s \times X_s}{V} \quad \text{2-4b}$$

Where: P_a *= total areal TSS productivity (g/m²/d)*
Q_m *= mixture discharge flowrate (L/d)*
Q_s *= supernatant discharge flowrate (L/d)*
X_m *= mixture TSS concentration (mg/L)*
X_s *= supernatant TSS concentration (mg/L)*
A_s *= surface area of reactor (cm²)*
P_v *= total volumetric TSS productivity (g/m³/d)*
V = reactor volume (L)

2.4.3 Sedimentation and Settleability

Sedimentation was quantified by various means during this research (Table 2-3). Measurements were conducted in 1-L Imhoff Cones alongside reactors or within reactors during their actual sedimentation periods so that they were subjected to the same conditions (e.g., light, temperature, cycle time). For volumetric comparisons, mixture SV (SM 2540F) was measured in Imhoff Cones after a 60-min sedimentation period, and any voids (i.e., usually < 1-2 mL) were visually estimated and subtracted from final SV (APHA 2005). Effects of light and container on sedimentation were also investigated (Sections 4.3 and 5.4.2, respectively).

Table 2-3. Summary of Sedimentation Estimates Obtained during this Research.

Thesis Section	Settling Container	Light	Volumetric Method	Gravimetric		Implication of Estimated vs. Actual Settleability
				Imhoff Method	*In-situ* Method	
3.1	Glassware	On			X	Accurate
3.2	Imhoff Cone	On	X		Periodically	Underestimated
3.3	Reactor	On			X	Accurate
4.3	Imhoff Cone	Off/On	X	X	X	Underestimated
5.4	Reactor	Off			X	Accurate

Gravimetric measurements were normalised based on culture density (i.e., settleability), and so they could be used to consistently compare reactors despite variation in biomass morphology (i.e., bulking). Two methods were investigated to quantify settleability: actual settleable TSS (X_b) (Imhoff Method, Equation 2-5a) and theoretical settleable TSS by difference from unsettleable

supernatant TSS (X_s) (*In-situ* Method, Equation 2-5b). These methods were significantly correlated for all samples ($r[178]=0.82$) with a higher correlation among samples of primary wastewater cultures ($r[88]=0.95$) probably because they generally had higher solids' contents and were less susceptible to low-level interference. Therefore, the Imhoff Method (Equation 2-5a) was used for cultures with low TSS (e.g., < 200 mg/L) for greater accuracy (Chapter 4), and the *in-situ* Method (Equation 2-5b) was used elsewhere because it was less time-consuming while still being reliable for higher TSS cultures.

$$S = 100 \times \frac{X_b}{X_m} \quad \text{(Imhoff Method)} \qquad 2\text{-}5a$$

$$S = 100 \times \left(1 - \frac{X_s}{X_m}\right) \quad \text{(\textit{In-situ} Method)} \qquad 2\text{-}5b$$

Where: *S = settleability (%)*
X_b = settleable TSS from a 1-L Imhoff Cone (Section 2.3.3) (mg/L)
X_m = mixture TSS concentration (mg/L)
X_s = supernatant TSS concentration (mg/L)

Sludge volume index is a hybrid volumetric/gravimetric calculation (mL/g) that can also be used to estimate settleability. However, it was not recommended for use in research applications due to its highly site-specific nature (Dick and Vesilind 1969); thus, it was not presented in herein.

2.4.4 COD and Nitrogen Species

Colorimetric measurements of COD and nitrogen species were made using a Hach DR/2000 or Hach Odyssey spectrophotometer. COD analysis in the range of 200-1,200 mg/L was similar to SM 5220D (APHA 2005) (Appendix B). Low range COD (< 150 mg/L), total nitrogen (TN), ammonia (NH_3), nitrate (NO_3^-), nitrite (NO_2^-), and (ortho)phosphate (PO_4^{3-}) analyses were conducted in accordance with Hach (2003) using standard reagents and a Hach DRB200 digester, where applicable. Samples for soluble analyses were filtered using 0.45 µm cellulose membrane filters prior to analysis.

High solids samples (i.e., from AS-inoculated cultures) often contained too much background colour for reliable spectrophotometric measurements. Therefore, these samples were filtered with 0.45 µm cellulose membrane filters before conducting NH_3, NO_3^-, and NO_2^- analyses in order to eliminate colour interference. A Sonics Vibra-Cell cell disrupter was used to homogenise high solids, total COD samples at 30-60% amplitude for 0.5-1 min to improve sample representativeness due to the occurrence of larger flocs in AS-inoculated cultures.

2.4.5 Turbidity

Turbidity was measured using a Hach 2100N or 2100P turbidimeter according to SM 2130B (APHA 2005). The appropriate calibration standard was checked to fall within 5% of the expected value before every use. Since turbidity is caused by suspended and colloidal material, it was hoped that it could be used as a surrogate measurement for various analyses (e.g., TSS, COD) after developing regression equations. However, this correlation was unreliable due to changes in light scattering from various particle sizes, shapes, and compositions (Kleizen et al. 1995; Sadar 1998). Therefore, turbidity was only regarded as a casual indication of culture dynamics (i.e., growth estimate). For example, turbidity increase of a culture between days 1 and 2 probably indicated an increase in biomass since properties were unlikely to change in 1 day, but comparing turbidity of two cultures operated differently was misleading due to likely differences in biomass characteristics.

2.4.6 Chl *a*

Photosynthetic pigments enable microalgae to harvest light energy. The three major classes of pigments are chlorophylls, carotenoids, and phycobilins. Chlorophyll (Chl) *a* is the preferred indicator for microalgal biomass quantification since it comprises 1 to 2% dw of all microalgae (APHA 2005) while Chl *b*, *c*, *d*, and *e* are accessory pigments which can augment light adsorption. Chl *a* was analysed by Cawthron Institute in Nelson, NZ according to Lorenzen (1967) and by NIWA Water Quality Laboratory in Hamilton, NZ according to SM 10200H (APHA 2005). Assuming 1.5% Chl *a* in microalgae, microalgal content was calculated according to Equation 2-6.

$$F_M = 100 \times \frac{(C/0.015)}{TSS} \qquad \text{2-6}$$

Where: F_M *= mass fraction of microalgae (%)*
C = Chl a concentration (mg/L)
TSS = total suspended solids concentration (mg/L)

Several sources of bias surrounded Chl *a* analysis. Firstly, Chl *a* contents vary depending on species physiology and abiotic factors (e.g., climate), which can result in perceived microalgal biomass differences among different microbial communities. Secondly, chlorophylls degrade into phaeopigments as communities age and cells die. Chl *a* measurements must be corrected for these phaeopigments since they have the same absorption peak. Thirdly, sample preparation and extraction solvent used significantly impact results (Biggs and Kilroy 2000; Ritchie 2006; Schagerl and Künzl 2007). Variation in Chl *a* among species was not resolvable since native, dynamic, multi-species communities were used as inocula. Influence from Chl *a* degradation and preparation techniques was minimised by NIWA Water Quality Laboratory through phaeo-pigment correction and maintaining consistent analytical procedures to the extent possible.

2.4.7 Energy

Heat of combustion (HC) was determined using an automatic adiabatic bomb calorimeter (Gallenkamp Autobomb, model CB-100). The calorimeter was calibrated using benzoic acid, cotton, and platinum wire to determine its heat capacity (Equation 2-7). Solids were oven-dried in porcelain crucibles at approximately 60 °C. A known mass of dried solids was ignited in an atmosphere consisting of 30 bar of pure oxygen. Then, HC was calculated using the corresponding temperature increase of a constant mass of water (Equation 2-8).

$$C = \frac{H_{acid} \times m_{acid} + H_{cotton} \times m_{cotton} + H_{wire} \times m_{wire}}{T_{final} - T_{initial}} \tag{2-7}$$

$$H_{sample} = \frac{C \times (T_{final} - T_{initial}) + H_{cotton} \times m_{cotton} + H_{wire} \times m_{wire}}{m_{sample}} \tag{2-8}$$

Where: C = heat capacity of calorimeter (J/K)
H = heat of combustion (J/g)
m = mass (g)
T = water temperature (K or °C + 273.15)

2.4.8 Lipids

Biomass harvested from each set of replicate experiments was ultimately combined to provide sufficient quantity for lipids analyses. It was dried in an incubator (Contherm Digital Series) at 60 °C for 12-72 h (i.e., dependent on sample size) since Zepka et al. (2008) found that this temperature was preferred for lipids preservation. International Accreditation New Zealand (IANZ) accredited lipid analyses were conducted by AsureQuality in Auckland, NZ. Total lipids were extracted with diethyl ether and petroleum based on SM 5B (IDF 1986) and 127A (IDF 1988) and SM 922.06, 950.54, 948.15, 954.02, 933.05, and 945.44 (AOAC 2005). Fatty acid methyl esters (FAMEs) were derived from a separate aliquot of sample using methanolic sodium hydroxide and boron trifluoride methanol according to SM 991.39 (AOAC 2005). Individual FAMEs were then grouped as polyunsaturated, monounsaturated, and saturated fatty acids (PUFAs, MUFAs, and SFAs) according to chemistry (i.e., > 1, 1, and 0 double bonds, respectively). Unidentified lipids were calculated via difference between total lipids and identified FAMEs. Yield of extractable material was determined and expressed by percent on a dry mass basis for fat (g fat/100 g sample) and on a dry fat basis for FAMEs (g FAME/100 g sample).

2.4.9 TKN and TP

Total Kjeldahl nitrogen (TKN) (SM 4500-N[B]) and total phosphorus (TP) (SM 4500-P[B+E]) were analysed according to APHA (2005). These IANZ-accredited tests were performed by Christchurch City Council (CCC) Laboratory at CWTP in Christchurch, NZ.

2.4.10 TC and TOC

Total carbon (TC) and total organic carbon (TOC) were analysed using a Teledyne Tekmar Apollo 9000 Combustion TOC Analyser according to SM 5310B (APHA 2005). Prior to analysis, a Sonics Vibra-Cell cell disrupter was used to homogenise TC and TOC samples at 60% amplitude for 1 min to improve sample representativeness due to the occurrence of larger flocs in AS-inoculated reactors. This sample preparation also was effective in reducing sedimentation of suspended biomass during lengthy analyses compared to undisrupted samples.

2.4.11 Microalgal and Microfaunal Taxonomy

Floc sizes were visually examined and measured for the longest dimension using a 2,000 X magnification, bright field light microscope (Olympus B $\times$ 50). Observations of microalgal and microfaunal species were compared to those of John et al. (2002) and Wiltshire and Broady (2008) for identification. Dr. Paul Broady, a microalgal taxonomist at UC, was also consulted as needed to identify unknown specimens. Photomicrographs were taken using an Olympus Camedia C-5060 digital camera.

2.4.12 Bacterial Taxonomy

The bacterial community composition of settled and supernatant biomass samples was determined at the end of select experimental runs using DNA-based methods. Hugenholtz (2002) reviewed this strategy and its applications in microbial ecology. Briefly, DNA was extracted from each sample and used to generate clone libraries (up to 95 clones/ sample) from polymerase chain reaction (PCR)-amplified, 16S ribosomal RNA (rRNA) genes as generally described by Smith et al. (2003). The cloned, 16S genes were sequenced and compared to existing records in GenBank and the Ribosomal Database Project (RDP) II using BLAST and Classifier programs, respectively. Taxonomic affiliations were determined based on > 97% sequence identity (BLAST) or 95% confidence level (Classifier). Relative abundances of phyla/classes/genera were calculated as percentage of sequences identified for each group within the gene library. For example, if 34 sequences were identified as Proteobacteria within a total library of 88 sequences, Proteobacteria comprised 39% of the library. This work was conducted by Dr. Susan Turner and Kristi Biswas, collaborators at the Microbial Ecology and Genomics Laboratory, School of Biological Sciences, University of Auckland, NZ.

2.5 Quality Assurance/Quality Control

Laboratory analyses were conducted according to standard methods (IDF 1986; IDF 1988; AOAC 2005; APHA 2005) and others as described above. Analytical instruments were properly calibrated and maintained in accordance with manufacturer's guidelines. Duplicates, standards, and blanks were regularly analysed to verify quality assurance/quality control (QA/QC) of laboratory results (Appendix B). Precision was monitored via relative percent difference (RPD) of duplicates (Equation 2-9). Accuracy was monitored via error of true and expected results of

prepared standards (Equation 2-10). Corrective actions such as resampling, reanalysing, and recalibrating instruments were performed, when possible, for QA/QC values exceeding ±25%.

$$\text{RPD}(\%) = 100 \times \frac{\text{original - duplicate}}{(\text{original} + \text{duplicate})/2} \tag{2-9}$$

$$\text{Error}(\%) = 100 \times \frac{\text{true} - \text{expected}}{\text{true}} \tag{2-10}$$

2.6 Climate Data

2.6.1 Ambient Climate Laboratory Conditions

Ambient climate laboratory studies were conducted in the Environmental Laboratory of UC's Civil and Mechanical Engineering building (E337). Air temperature and humidity were generally 20-21 °C and 40-50%, respectively. Water temperature and PAR of cultures, which varied somewhat between experiments, were also monitored.

2.6.2 Controlled Climate Laboratory Conditions

Setpoint Selection. Controlled climate laboratory studies were conducted in a temperature controlled room in the Environmental Laboratory of UC's Civil and Mechanical Engineering building (E337B). Global radiation (i.e., light intensity and duration) and air temperature for two climatic conditions were based on historic means obtained from NIWA (2008) for Christchurch, NZ from 1998 to 2007 (Table 2-4). Winter values were calculated using June through August daily means, and summer values were calculated using December through February daily means. Day length was fixed to a mean seasonal value for Christchurch, NZ using a programmable controller. PAR was approximately 50% of global radiation, and it was relatively consistent in that proportion both diurnally and seasonally (Hall et al. 1993). Thus, PAR was calculated from global radiation using mean day length (Equation 2-11). Since light intensity could not be easily adjusted during the day to simulate natural conditions, a constant value was used over the entire light period that amounted to the mean total daily radiation.

Table 2-4. Key Climatic Conditions for Christchurch, NZ.

Parameter	Season	
	Winter	Summer
Global Radiation [Range] ($MJ/m^2/d$)[(a)]	6.1 [5.4-7.0]	20.2 [17.2-22.9]
Daytime PAR [Range] ($\mu mol/m^2/s$)[(a,b)]	408 [292-604]	877 [699-1,059]
Day Length [Range] (h/d)	9.6 [9.0-10.5]	14.7 [13.8-15.4]
Air Temperature [Range] (°C)[(b)]	6.5 [1.2-11.8]	16.4 [11.1-21.7]
Relative Humidity [Range] (%)[(a)]	86 [81-90]	76 [65-84]

(a) Mean daily data for Christchurch, NZ from 1998-2007 (NIWA 2008).
(b) Based on global radiation values for mean day length.

$$PAR = \frac{0.5 \times GR \times CF \times 10^6}{DL \times 3600} \quad \text{2-11}$$

Where: *PAR = photosynthetically active radiation (μmol photon/m²/s), 400-700 nm*
GR = global radiation (MJ/m²/d)
CF = 4.6 conversion factor for daylight and metal halide lamps (Hall et al. 1993)
DL = day length (h/d)

Setpoint Deviations. Air conditioning difficulties prevented air temperature from reaching the 6.5 °C setpoint. During the light period, the 400-W bulbs provided a large heat load that was only able to be partially dissipated by the dedicated cooling coil and supplemental air fans. In addition, ice gradually built up on the cooling coil which decreased its efficiency and caused room temperature to rise. Thus, direct heating using a fan heater was routinely required to melt ice off the coil and restore its performance. Ice was also placed in the room in buckets, as needed, to improve cooling and maintain consistent temperatures. Despite these efforts, temperature for the Cold Studies (i.e., M=13, SD=3 °C [Section 4.1.3]) exceeded Christchurch's winter mean range (i.e., 1.2-11.8 °C [1998-2007]). In light of this, higher temperature and PAR values (rather than the means) were accordingly selected for summer conditions to provide greater difference between the two climates for comparison purposes. Climatic conditions were therefore referred to as 'Cold' and 'Warm' rather than 'Winter' and 'Summer' due to these changes.

When the controlled climate studies began in July 2008, 86% relative humidity (RH) was not able to be consistently maintained. A lower value of approximately 60% RH was achievable and, therefore, set as a constant. A new humidifier was installed in September 2008, but because two replicates had already been subject to the reduced setpoint, 60% RH remained constant through completion of the controlled climate experiments. Regardless of this deviation, humidity was not expected to have a significant impact compared to other factors since it has not been linked to microbial growth in wastewater applications.

2.7 Statistical Analyses

Data tabulation, computations, and graphing were performed using Microsoft Excel. Uncertainty was conveyed through summary statistics and graphs (using error bars) as one standard deviation (SD) of the mean (M) for n observations. Independent means (e.g., culture end values) were compared using a two-tailed t test or an analysis of variance (ANOVA) (F statistic). Dependent means were compared using a paired t test where applicable. The t statistic or F statistic, degrees of freedom (df) (both between and within groups for ANOVA), and significance level (p) were reported based on American Psychological Association (APA) format (Kahn 2010). Differences were accepted as significant for $p < 0.05$ unless stated otherwise. The strength of association between two parameters was reported using Pearson's correlation coefficient (r) and df.

Comparison of temporally dependent data (i.e., repeated measures of more than two non-steady state systems over time [Chapter 4]) required more robust software for statistical analysis. Common tools such as multiple linear regression and ANOVA were not applicable because they required independent observations. Therefore, a linear mixed-effects model (LMM) was used for the analysis because it enabled these dependent datasets to be modelled with fixed and random effects (Stauffer 2008). LMM analysis was carried out by James Dawber (BSc[Hons] in Mathematics and Statistics at UC) using R, a statistical computing software with its own programming language (R Development Core Team 2008). The LMM incorporated fixed effects of monitoring day and treatment as well as the random effect of variation between replicates. Mr. Dawber provided the intercepts, slopes, and overall significance (i.e., yes/no for $p < 0.05$) of various trends from the LMM as summarised by the researcher (Chapter 4).

2.8 Health and Safety Approach

The recommended health and safety precautions detailed by UC (2007) were adhered to for all research conducted. The researcher was also informed of safety precautions for the Environmental Laboratory (within Civil and Natural Resources Engineering), Phycology Laboratory (Physical Containment 2 laboratory within the School of Biological Sciences), Chemical and Process Engineering laboratories, and CWTP including sign-in, sampling locations, and washroom facilities. The researcher was properly trained on the use of any new equipment and laboratory methods before they were adopted. As municipal wastewater is a biological hazard, the researcher was diligent about handwashing and personal protective equipment usage. She also had received prior training in first-aid, cardiopulmonary resuscitation, fire extinguishers, and Hazardous Waste Operations and Emergency Response.

Chapter 3: Preliminary Experiments Using Ambient Climate Laboratory Reactors

3.1 *Batch Experiments*

3.1.1 Microalgal Inoculum Selection Experiment

The best source of native microalgae from oxidation ponds for laboratory reactor experiments using primary wastewater was examined. Three, 2-L reactors were established according to Section 2.2.1. Each reactor received 150 mL primary wastewater and either 1 L of Oxidation Pond 1 (P1) wastewater (culture C1) or 1 L of Oxidation Pond 6 (P6) wastewater (cultures C6 and C6F). Only P6 inoculum for C6F was filtered through 80-µm nylon mesh to remove larger microfauna and detritus.

Microalgal taxonomy of the two oxidation pond inocula and three mixed cultures was determined by light microscopy. On day 1, P6 had greater microalgal diversity (i.e., more species present) than P1, which had mostly euglenoids and bacteria (Table 3-1). After 8 d of batch growth and continuous mixing, visual observations showed that the cultures had developed similar microalgal communities despite the differences in their P1 and P6 inocula. Primary

Table 3-1. Microalgal Taxa of Batch-Grown Cultures of Oxidation Pond and Primary Wastewaters.

Class: Genus and Species	Oxidation Pond Inoculum (Day 1)		Reactor Culture (Day 8)		
	P1	P6	C1	C6	C6F
Chlorophyceae: *Coelastrum* sp.		X			
Chlorophyceae: *Dictyosphaerium* sp.			X		
Chlorophyceae: *Micractinium* sp.			X		
Chlorophyceae: *Oocystis* sp.		X			
Chlorophyceae: *Pediastrum* sp.		X			
Chlorophyceae: *Scenedesmus* sp.			X	X	X
Chlorophyceae: cf. *Eudorina* sp.		X	X	X	X
Chlorophyceae: *Closterium* cf. *aciculare*					X
Chlorophyceae: *Chlorococcum* sp., *Chlorella* sp., *Choricystis* sp., and/or fragmented *Micractinium* sp.		X	X	X	X
Euglenophyceae: *Lepocinclis* cf. *texta*	X	X			
Euglenophyceae: Unknown	X	X		X	
Bacillariophyceae: *Nitzschia* sp.			X		X

Note: C1 = 1 L P1 (unfiltered) and 150 mL primary wastewater; C6 = 1 L P6 (unfiltered) and 150 mL primary wastewater; C6F = 1 L P6 (80-µm filtered) and 150 mL primary wastewater.

wastewater and ambient laboratory climatic conditions had selected for the growth of species suited to the environment provided. Although some of the microalgal genera identified on day 8 were not initially identified on day 1, it was reasonably assumed that these taxa were also present on day 1, but not observed, due to their low concentration in the inocula, limited identification experience of the examiner, and the cursory nature of the identification. Overall, the predominant microalgal class within the reactors on both sampling days was Chlorophyceae including the colonial genera *Scenedesmus* and other green microalgae, which agreed with observations by others for treated municipal wastewaters in NZ (Craggs 2001; Garden 2005; Novis 2007; Wiltshire and Broady 2008).

Analysis of COD indirectly measured the organic content of the cultures (APHA 2005) to provide an indication of microbial biomass growth. On day 8, COD was 769, 957, and 1,047 mg/L for C1, C6, and C6F, respectively, indicating 10-36% greater biomass in C6F compared to the other cultures. Filtered P6 wastewater was therefore chosen as a microalgae inoculum for subsequent investigations because:

1) it had a greater quantity of diverse microalgae;
2) it would be less impacted than P1 in case of operational issues at CWTP since it is further downstream in the treatment process; and
3) filtering large microfauna (e.g., algivores) from the inoculum enhanced microbial biomass growth in C6F compared to unfiltered C6.

3.1.2 Activated Sludge Addition Experiments

Microalgae and bacteria have a symbiotic relationship that exploits metabolic CO_2/O_2 exchange–microalgae supply photosynthetic O_2 to aerobic bacteria while bacteria supply respiratory CO_2 to microalgae (Humenik and Hanna 1970). This relationship indicates that microbial growth could be enhanced by optimising proportions of microalgae and bacteria. Furthermore, mixed *Chlorella*-AS cultures have demonstrated good biomass settleability and wastewater treatment (Humenik and Hanna 1971; Aziz and Ng 1992; Gutzeit et al. 2005), so several experiments were conducted to examine the value of AS addition to microalgal wastewater cultures in Christchurch, NZ.

3.1.2.1 Effects on Microalgal Growth

An experiment was conducted using P6 wastewater and various concentrations of AS to observe its effects on microalgal growth. Three, 1-L flasks were inoculated with P6 wastewater and 0, 5, and 10% (v/v) AS and set up according to Section 2.2.1. The 0% AS culture, which was essentially P6 wastewater only, served as the control.

Visually, all of the cultures became greener over time indicating that photosynthesis was occurring and microalgal biomass was increasing. Microalgae in the 0% AS culture rapidly

metabolised dissolved inorganic carbon (DIC) and other nutrients from P6 wastewater (and CO_2 from air) for growth as demonstrated by increasing COD over time (Figure 3-1). However, COD of 5 and 10% AS cultures remained fairly stable probably because bacterial starvation and AS degradation were occurring due to limited nutrients in P6 wastewater. Meanwhile, microalgae were growing in support of the visual observations. Because COD analysis made no distinction between living, non-living, microalgal, and/or bacterial biomass, it was possible that microalgal biomass replaced bacterial biomass in these cultures resulting in relatively unchanged COD.

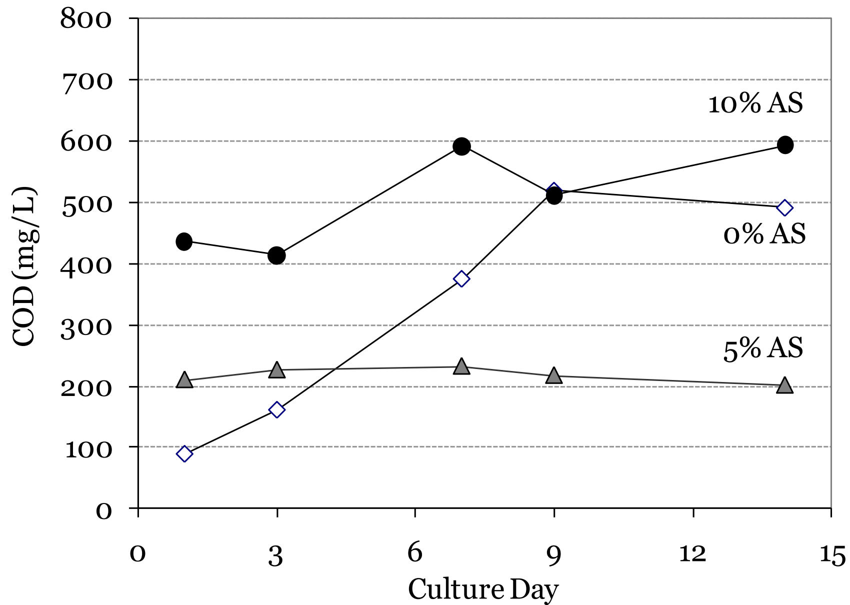

Figure 3-1. COD of Batch-Grown Cultures of P6 and AS Wastewaters over Time.

DIC switched between several different chemical species: carbonate (CO_3^{2-}), bicarbonate (HCO_3^-), and carbon dioxide (CO_2) (Equation 3-1) in response to pH changes. As microalgae used CO_2 for photosynthesis, hydroxide (OH^-) accumulated in the culture which increased its pH. Periodic measurements showed that the 0% AS culture had relatively high pH and DO (i.e., 9.4 SU and 12.0 mg/L) reflecting the effects of photosynthesis, which can inhibit microalgal growth (Richmond 2004). The 5 and 10% AS cultures had lower (neutral) pH (i.e., 6.8-7.0 SU) and DO (i.e., 7.2-8.2 mg/L) than the control presumably from reduced photosynthesis due to shading and/or from greater pH/DO buffering from organics and ions in AS.

$$CO_3^{2-} + H_2O \Leftrightarrow HCO_3^- + OH^- \Leftrightarrow CO_2 + 2OH^- \qquad 3\text{-}1$$

In summary, the 0% AS culture had excessively high pH and DO, and the 5 and 10% AS cultures were probably overloaded with AS resulting in insufficient oxygenation and AS degradation. Therefore, AS inoculum was reduced to approximately 2% (v/v) AS, and P6 inoculum was standardised to ensure consistent ratios of P6 and AS microbes in future experiments.

3.1.2.2 Effects on Microalgae Harvesting

Microalgal settleability impacts the economics of harvesting by sedimentation as greater settleability improves biomass recovery and reduces operating cost. Using AS (i.e., a waste product) rather than purchased chemicals (e.g., polymers or inorganic salts) to enhance settleability of P6 wastewater-derived biomass could improve its harvestability and sustainability for microbial biomass production. Therefore, tests were conducted using 2-L beakers to determine if AS could be used as a rapid microalgal flocculant for P6 wastewater. This concept was a novel idea suggested by Franz Resl at CWTP. Sludge ratio (0-22 mg AS/mg P6 wastewater), mixing time (1 or 20 min), and separation method (30-min sedimentation or 1-min air flotation) were all tested to determine their effects on mixture harvestability. The AS and P6 wastewaters, as characterised in Table 3-2, were used within 2 h of sampling from CWTP and combined in various quantities to obtain an array of mixtures that could be examined to optimise microbial proportions for sedimentation (Table 3-3).

Table 3-2. Characteristics of P6 and AS Wastewaters.

Parameter	Value
P6 Wastewater	
TSS (mg/L)	45
Temperature (°C)	23.7
Activated Sludge	
TSS (mg/L)	3,180
30-min Settled Volume (mL/L)	450
Sludge Volume Index (mL/g)	141

Table 3-3. P6 and AS Wastewater TSS Contributions to Mixtures.

Culture	AS:P6 TSS Ratio (mg/mg)	P6 Portion of TSS (mg/L)	AS Portion of TSS (mg/L)	Mixture TSS (mg/L)
C1	0.0	45	0	45
C2	0.2	45	10	55
C3	1.1	45	50	95
C4	2.2	44	96	140
C5	4.4	43	188	231
C6	8.8	40	356	396
C7	22	35	760	795

Testing Apparatus. The testing apparatus and procedure conformed to standard D2035-80 (ASTM International 2003) except that the “flash mix” (i.e., short, rapid mixing) step was omitted to avoid cell damage at high shear stress (Richmond 2004). Up to six tests were conducted simultaneously. Beakers contained 1 L P6 wastewater and received 0-314 mL AS (Figure 3-2). Immediately following AS addition, mixing was conducted for 1 or 20 min at 25 rpm.

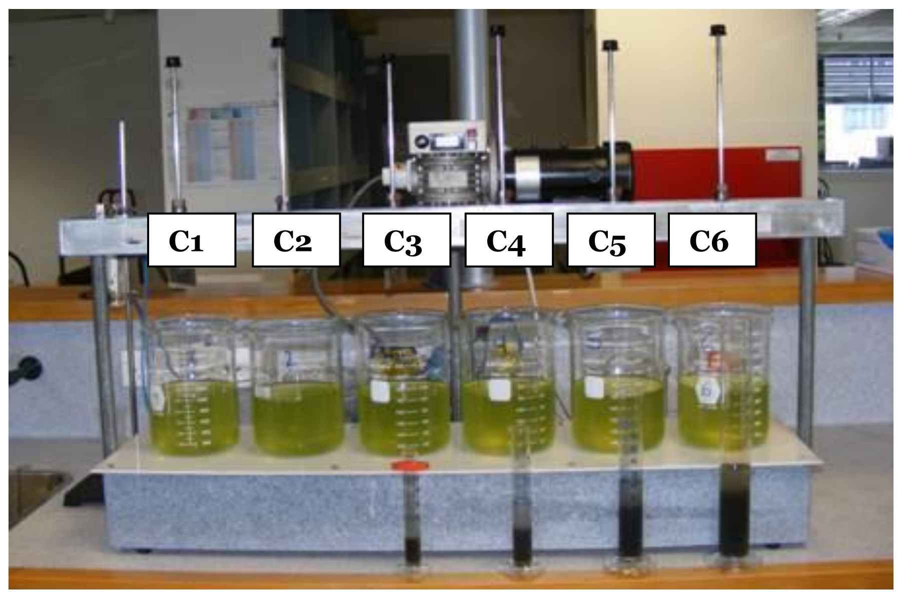

Figure 3-2. Testing Apparatus Showing P6 Wastewater in Beakers before AS Addition.

Solid-Liquid Separation. Following mixing, sedimentation and air flotation were used for solid-liquid separation. Sedimentation occurred for 30 min before a supernatant sample was collected from halfway between the water surface and settled solids. Air flotation was performed for 1 min at 6 L/min using compressed air through a flowmeter and basic aquarium aerators (i.e., air stone discs or perforated, coiled tubing), which were installed at the base of the beakers. Following aeration, mixtures rested for an additional 1 min before samples were collected from halfway between the water surface and aerator.

Results and Discussion. Supernatant TSS was determined after 20-min mixing and 30-min sedimentation (i.e., C'X'a data) for all AS:P6 ratios and also for 1-min mixing and 30-min sedimentation (i.e., C'X'b data) for three AS:P6 ratios (Figure 3-3). Longer mixing time clearly enhanced settleability as evident by lower supernatant TSS. Conditions that produced the lowest supernatant TSS were 22 mg AS/mg P6 ratio, 20-min mixing, and 30-min sedimentation (C7a [Figure 3-3]). However, since P6 portion of TSS decreased as AS:P6 ratio increased (Table 3-3), the greatest *improvement* in supernatant TSS reduction was actually achieved using 8.8 mg AS/mg P6 ratio, 20-min mixing, and 30-min sedimentation (C6a [Figure 3-3]). Sample C6a had only 74% of the initial P6 portion of supernatant TSS remaining compared to 83% remaining for C7a. Controls (i.e., without AS added [C1]) also demonstrated reduction in supernatant TSS (i.e., ≤ 26%) due to some natural settling of the suspensions.

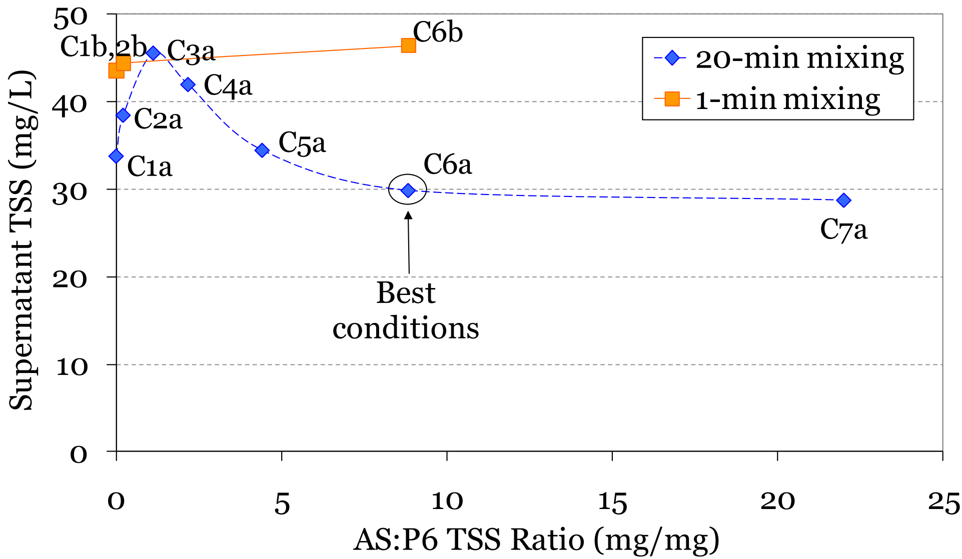

Figure 3-3. Effect of AS Ratio and Mixing on 30-min Sedimentation.

Air flotation using the available equipment (Figure 3-4) was not effective at enhancing solid-liquid separation. Supernatant TSS increased 2- to 6-fold except for the control (C1c), which was unchanged (Appendix C). Air bubbles were probably too large and caused excessive turbulence to be representative of dissolved air flotation (DAF) typically employed in solid-liquid separation applications. Future DAF tests conducted in this context could utilise an air flotation cell (Eckenfelder 2000) for a more accurate assessment. Nonetheless, sedimentation tests can still provide a conservative measure of the amount of solids that could be removed by DAF since flocs can generally be made to float faster than they can settle (Batchelor 1967).

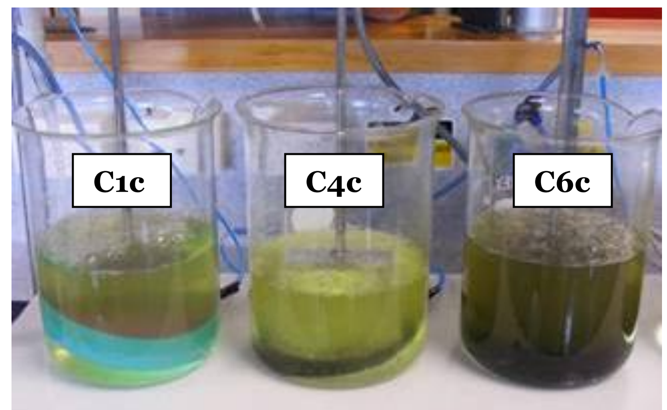

Figure 3-4. Setup of Solid-Liquid Separation Tests Using Air Flotation.

In summary, AS addition to P6 wastewater did not provide a clear benefit over natural settling of P6 wastewater. Therefore, AS was not pursued as a rapid microalgal flocculant for P6 wastewater. However, results suggested that biomass settleability could further improve with prolonged mixing conditions, so this avenue was re-examined in subsequent experiments.

3.2 Continuous Stirred Tank Reactor Experiment

3.2.1 Experimental Design

Two replicate laboratory reactors (R1 and R2) were inoculated with P6 wastewater, continuously (drip) fed with primary wastewater (Section 2.1), and operated as CSTRs (Section 2.2.2) as summarised in Table 3-4. Samples of mixture were collected for analyses approximately 2 h into the 12-h light period. This experiment was designed to scale-up the batch reactor experiments and examine the microbial behaviour under a continuous operating regime.

Table 3-4. CSTR Operating Parameters.

Parameter	Value	Remarks
Reactor Volume (L)	25	
Reactor Depth (cm)	35	
HRT (d)	7	2.5 mL/min flowrate
PAR (μmol/m²/s)	390	At water surface for 12 h/d
Water Temperature (°C)	21 ± 1	

3.2.2 pH and DO Concentrations

Absorption of atmospheric oxygen, photosynthetic oxygenation, and respiration impacted DO levels of the cultures. Absorption is continuous (except at supersaturated DO levels) and depends on system hydraulics and DO saturation. During the light period, microalgal photosynthesis also contributed oxygen to the culture (and depleted CO_2) to cause diurnal oscillations in DO from 0-6.0 mg/L and pH from 6.9-8.0 SU (Figure 3-5 and Table 3-5). Variability between the replicate CSTRs in these magnitudes was attributed to natural divergence of the microbial communities (Matheson et al. 2008).

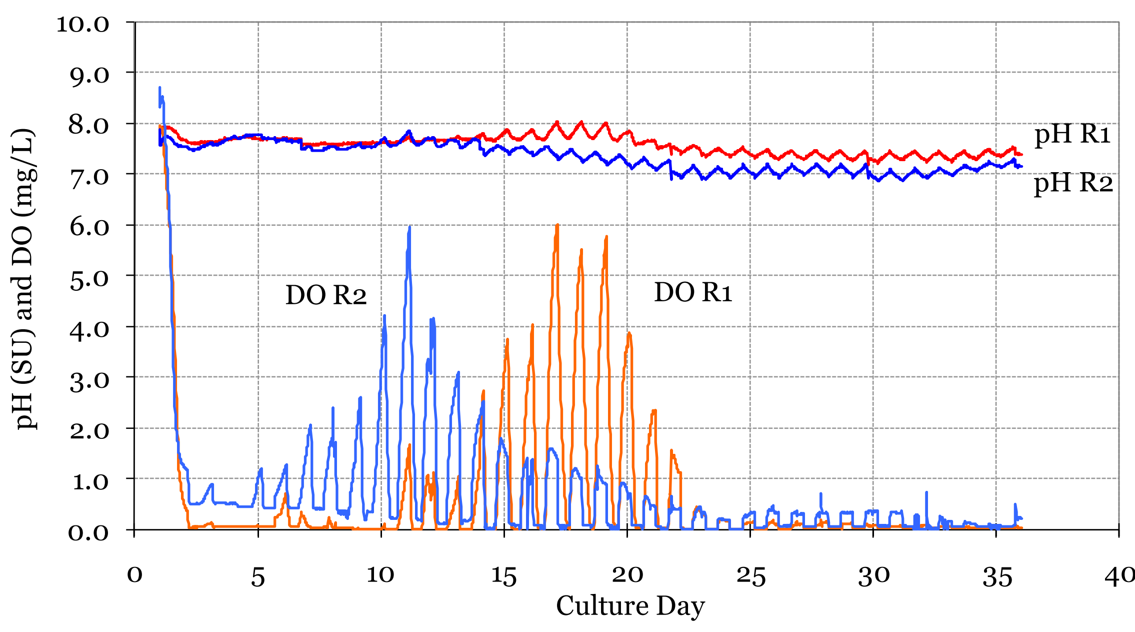

Figure 3-5. pH and DO Measurements of CSTR Cultures over Time.

Table 3-5. Summary of pH and DO Measurements of CSTR Cultures.

Parameter	CSTR	Unit	Concentration		
			Minimum (after Day 1)	Maximum (after Day 1)	*M* ± *SD*
pH	R1	SU	7.2	8.0	7.6 ± 0.2
	R2	SU	6.9	7.8	7.3 ± 0.3
DO	R1	mg/L	0.0	6.0	0.6 ± 1.4
	R2	mg/L	0.0	6.0	0.7 ± 1.2

Some of the DO trends over time (Figure 3-5) may be explained by the microbial growth. Initially, microalgae in the CSTRs were probably in lag growth and photosynthesising minimally until day 5-6 as evident by the low DO (i.e., < 1 mg/L). This period of low growth resulted from self-organisation and physiological adaptation to new environmental and nutritional conditions (Madigan et al. 2000; Richmond 2004). Then, microalgae appeared to commence exponential growth based on the increase in DO (Figure 3-5). After day 23 or 3 HRTs, DO and pH (and turbidity [Section 3.2.3]) had stabilised and reduced indicating decreased photosynthesis.

3.2.3 Biomass Production and Settleability

Turbidity was casually regarded as an estimate of suspended material (i.e., biomass) (Section 2.4.5). Increasing turbidity (and DO from microalgal photosynthesis) suggested that rapid microbial growth occurred until day 20-22. A stable condition subsequently continued through the end of the monitoring period as indicated by the stable turbidity (Figure 3-6a) and DO (Figure 3-5) trends. Although the reactors were designed as CSTRs without biomass recycle, some of the biomass was retained at the low, continuous discharge of 2.5 mL/min because it was partially obstructed by the overflow port.

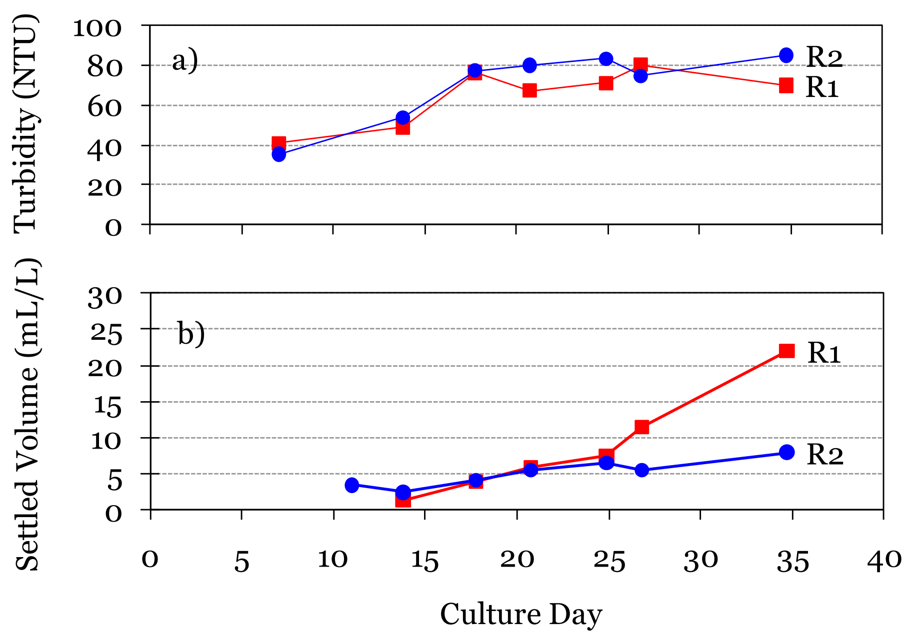

Figure 3-6. Turbidity (a) and Settled Volume (b) of CSTR Cultures over Time.

Biomass settleability was inferred from SV in conjunction with periodic supernatant and mixture TSS analyses. The two CSTRs displayed similar settleability until day 25, but then large differences in SV occurred on days 27 and 35 (i.e., 11.5/5.5 and 22/8.0 mL/L, respectively, for R1/R2) (Figure 3-6b). Culture R1 was more settleable than R2 (i.e., 57 vs. 44% settleability, respectively, on day 27), but this difference did not entirely account for SV variations since turbidity (i.e., biomass) was comparable on both days (Figure 3-6a). This discrepancy indicated that dissimilar floc morphologies may have resulted from divergence of the microbial communities over time (Matheson et al. 2008), which would have influenced the compressibility and settleability of the biomass (Jin et al. 2003).

3.2.4 Biomass Harvesting

Settled biomass from the CSTRs was combined into a bucket and dewatered by gravity sedimentation and decantation. Several iterations were performed over a 24-h period to finally obtain 0.8 L concentrated biomass. The dewatered biomass had a moisture content of 98.4% (i.e., *SD*=0.0%), and it was highly organic (i.e., *M*=82.4, *SD*=0.4% VS). The 1.6% TS content was within the range typically achieved by sedimentation (i.e., 1-3% TS) (Benemann and Oswald 1996; Uduman et al. 2010). In total, approximately 13 g TS was recovered from both CSTRs. Final TSS of 170 and 160 mg/L for R1 and R2, respectively, indicated 4 g TSS in the mixtures, and the balance of solids (to acquire a total of 13 g TS) was adhered to the CSTRs' walls as microbial biofilm. Since the reactors have a greater surface area to volume ratio (16) compared to large-scale systems (generally < 10), any accumulated biofilm would probably have a smaller contribution to harvested mass. Ultimately, sedimentation resulted in a 63-fold volumetric reduction, but secondary dewatering would further improve the efficiency of biomass conversion (e.g., 10-20% TS required for anaerobic digestion).

3.3 Sequencing Batch Reactor Experiment

3.3.1 Experimental Design

Microbial reactors were examined from a wastewater treatment perspective to confirm their credence for sustainable biomass production (i.e., nutrient recycling from 'waste' water [Section 1.4.5]). Two replicate reactors were inoculated with 18.4 L P6 wastewater and 2.8 L primary wastewater (Section 2.1), fed with primary wastewater, and operated as SBRs (Section 2.2.2) according to Table 3-6. Microbial biomass was continually accumulated during each RP with solids removed only by sampling and discharge of unsettled biomass in supernatant resulting in increasing SRT from 9-80 d over the monitoring period. Mixture was sampled at the start and end of each 3-d RP and discharged supernatant was also sampled from each SBR in an alternating pattern. For instance, SBR1 was sampled for RPs 1, 3, 5… and SBR2 was sampled for RPs 2, 4, 6…

Table 3-6. SBR Operating Parameters.

Parameter	Value	Remarks
Reactor Volume (L)	21	
Reactor Depth (cm)	30	
Reaction Phase (d)	3	
HRT (d)	9	7 L exchanged every 3 d
SRT (d)	9-80	Increased over time
PAR (μmol/m²/s)	516	At water surface for 12 h/d
Water Temperature (°C)	25 ± 3	*M* ± *SD*

3.3.2 Wastewater Treatment

3.3.2.1 Ammonia Concentrations

Ammonia is of key importance to municipal WTPs since it is a common contaminant that can be difficult to remove (Alley 2007). Ammonia constituted 68% of TN on average in the primary wastewater (Table 2-1). Effluent ammonia averaged 5.5 mg/L (as N) through RP 8, which represented a reduction of 82% on average using a 9-d HRT (Figure 3-7). Nunez et al. (2001) found that 25-33% of TN removed by a microalgal wastewater treatment system was converted into microbial proteins, and the remainder was volatilised from ammonia into nitrogen gas at a pH of 8.6-9.7 SU. Because nitrate was < 3 mg/L and nitrite was not detected (i.e., < 2 mg/L) (Appendix C), ammonia reduction was indicative of wastewater treatment.

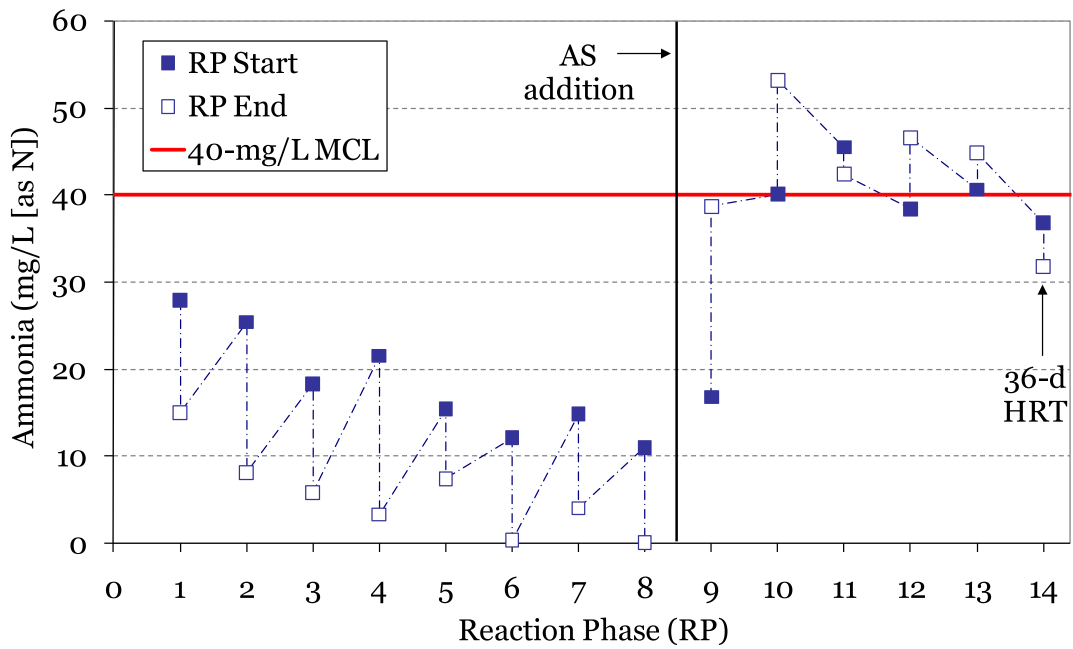

Figure 3-7. Ammonia Concentrations at Start and End of Each RP.

(Note: results of replicates sampled at alternating RPs shown; each RP was 3 d except RP 14, which was 12 d.)

After reaching stable conditions (Section 3.3.3.1), an attempt was made to improve biomass flocculation and settleability using AS since it had demonstrated improvements at prolonged mixing (Section 3.1.2.2). Therefore, 2.5 L AS (approximately 4-fold concentrated, 12% v/v, or 1,860 mg/L TSS) and 4.5 L primary wastewater were added to each SBR at the start of RP 9. Regular operating conditions (i.e., fed-batch primary wastewater with 9-d HRT) resumed following AS addition. Anaerobic (Section 3.3.2.2) and highly reducing conditions (based on H_2S odour) resulted within the SBRs. Subsequent breakdown and solubilisation of intracellular organic nitrogen (e.g., protein and nucleic acids) and/or EPS caused elevated ammonia levels (Figure 3-7). Cui (2004) also found that ammonia increased following addition of solubilised sludge to a laboratory reactor treating synthetic wastewater in anoxic conditions, and that mechanical aeration was required to oxidise ammonia to nitrate. Nitrification was inhibited by lack of oxygen, and the pH (i.e., typically < 8.0 SU) prevented volatilisation. Consequently, ammonia was removed very slowly beginning with RP 9, primarily by dilution with primary wastewater at the start of each RP and also through some microbial assimilation. At the end of the study, RP 14 was extended from 3 to 12 d (i.e., 36-d HRT) to monitor wastewater treatment over a longer period. Ammonia results for RP 14 suggested that microbial assimilation was still occurring to some extent, and that ammonification had decreased. Overall, microbial biomass grown in fed-batch SBRs on a mixture of P6 and primary wastewaters from CWTP showed effective ammonia removal and met the Canterbury Regional Council (CRC) Resource Consent's maximum contaminant level (MCL) of 40-mg/L for wastewater discharge (per CRC051724 [Ocean Outfall]), but wastewater treatment was not maintained following AS addition.

3.3.2.2 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 were followed by gradual decreases during the dark period that caused diurnal fluctuations. Comparison of pH and DO measurements from both SBRs revealed similar trends and magnitudes, indicating that strong replicates had been established (Figure 3-8a). The pH was higher with greater diurnal fluctuation pre-AS addition (i.e., 7.0-10.5 SU) compared to post-AS addition (i.e., 6.9-8.1 SU) (Figure 3-8a; Table 3-7). This trend was also observed for DO (i.e., maximum of 26 mg/L fell to 0.9 mg/L) (Figure 3-8b; Table 3-7). These changes were likely due to reduced microalgal photosynthesis caused by AS shading and/or ammonia inhibition (Section 3.3.3.2), greater buffering from organics and ions present in AS, and increased oxygen demand (i.e., for bacterial respiration and oxidation of organic matter). Consequently, microalgae could not produce enough DO to meet the elevated oxygen demand, the culture became anaerobic, and biomass decreased (Section 3.3.3).

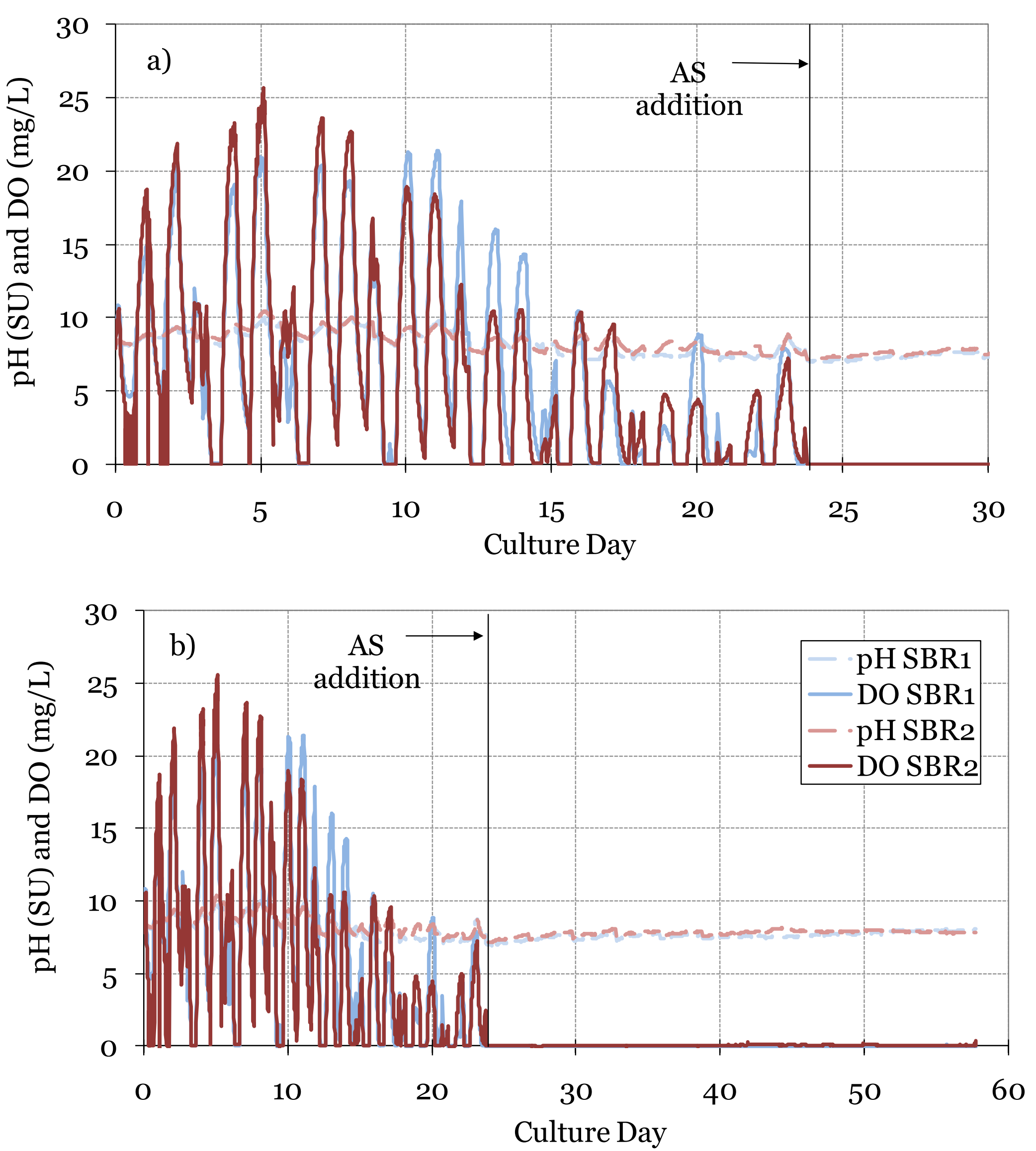

Figure 3-8. Continuous pH and DO Measurements of Replicate SBRs for a) Culture Days 0-30 and b) Culture Days 0-60.

Table 3-7. Summary of pH and DO Measurements of Replicate SBRs Recorded at 15-min Intervals.

Parameter	**RP Period**	**Concentration**		
		Minimum	**Maximum**	***M* ± *SD***
pH (SU)	Light	7.0	10.5	8.6 ± 0.7
	Dark	7.1	10.4	8.3 ± 0.7
	Light (w/ AS)	7.0	8.1	7.7 ± 0.2
	Dark (w/ AS)	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/ AS)	0.0	0.9	0.0 ± 0.0
	Dark (w/ AS)	0.0	0.1	0.0 ± 0.0

Note: 2.5 L AS added to each SBR (n=2) on day 24 of 58.

3.3.3 Biomass Production and Settleability

3.3.3.1 COD and TSS Concentrations

Analysis of COD and TSS was conducted to estimate biomass (Section 2.4.2). Mixture COD and TSS increased up to 105 and 152%, respectively, over each 3-d RP as microbial biomass grew during RPs 1-8 (Figure 3-9). By the end of RP 8, these concentrations were fairly stable (i.e., 624±9 mg/L COD and 432±9 mg/L TSS for RPs 6-8). Addition of (organic) solids in AS to the cultures at the start of RP 9 increased levels to approximately 2,200 mg/L TSS and 3,200 mg/L COD. Following these initial spikes, COD decreased by 5% and TSS by 3% on average for each RP during RPs 9-14 despite good settleability (Section 3.3.3.2). COD likely decreased from dilution by primary wastewater and by oxidation of organic matter using atmospheric and photosynthetic oxygen. TSS likely decreased from net biomass loss and solubilisation of organic matter probably due to death and degradation of aerobic bacteria once anaerobic conditions dominated following the AS addition.

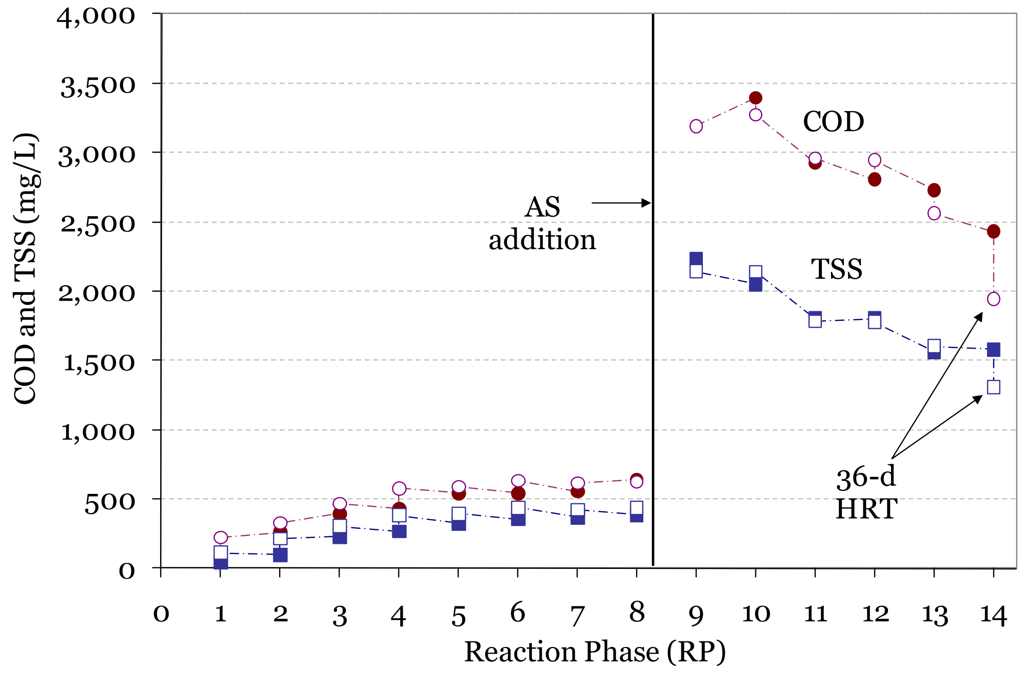

Figure 3-9. COD and TSS Concentrations at Start and End of Each RP.

(Note: results of replicates sampled at alternating RPs shown; each RP was 3 d except RP 14, which was 12 d; filled symbols show mixture values at RP starts and hollow symbols at RP ends.)

3.3.3.2 Microalgal Growth and Settleability

Chl *a* of the biomass reflected its microalgal content (Section 2.4.6). Greater Chl *a* at the end compared to the start of an RP signified an increase in microalgae concentration between RPs 1-8 (Figure 3-10). However, microalgal growth was severely inhibited following AS addition. Minimal growth was observed between mixture RP start and end samples in RPs 9-14, and Chl *a* generally decreased over time probably from dilution by primary wastewater (Figure 3-10).

Average mixture RP end Chl *a* reduced from 3.3 to 2.3 mg/L from RPs 3-8 to 9-14. Above a pH of 8.0 SU, ammonia > 28 mg/L (as N) has been shown to impair microalgal growth and photosynthesis (Abeliovich and Azov 1976). Following AS addition, ammonia was 32-53 mg/L (as N) (Figure 3-7), and pH was 6.9-8.1 SU (Table 3-7), so ammonia toxicity may have occurred. Growth could have also reduced as a result of shading by AS. In contrast, Miller et al. (1977) found that seeding microalgal-wastewater cultures with settled sludge (23% v/v) maximised Chl *a* compared to unseeded cultures (i.e., 9.5 vs. 5.2 mg/L) probably due to the added nutrients, amenable pH (i.e., < 8.0 SU), and greater light supply. Overall greater Chl *a* values by Miller et al. (1977) compared to SBR1/2 reflected the culture conditions (i.e., 21-d batch growth, continuous 10^4 erg/cm^2/s light, 27.5 °C temperature), and differences between AS-seeded and unseeded cultures were consequently not as substantial (i.e., SBR1/2 with and without AS) in this study.

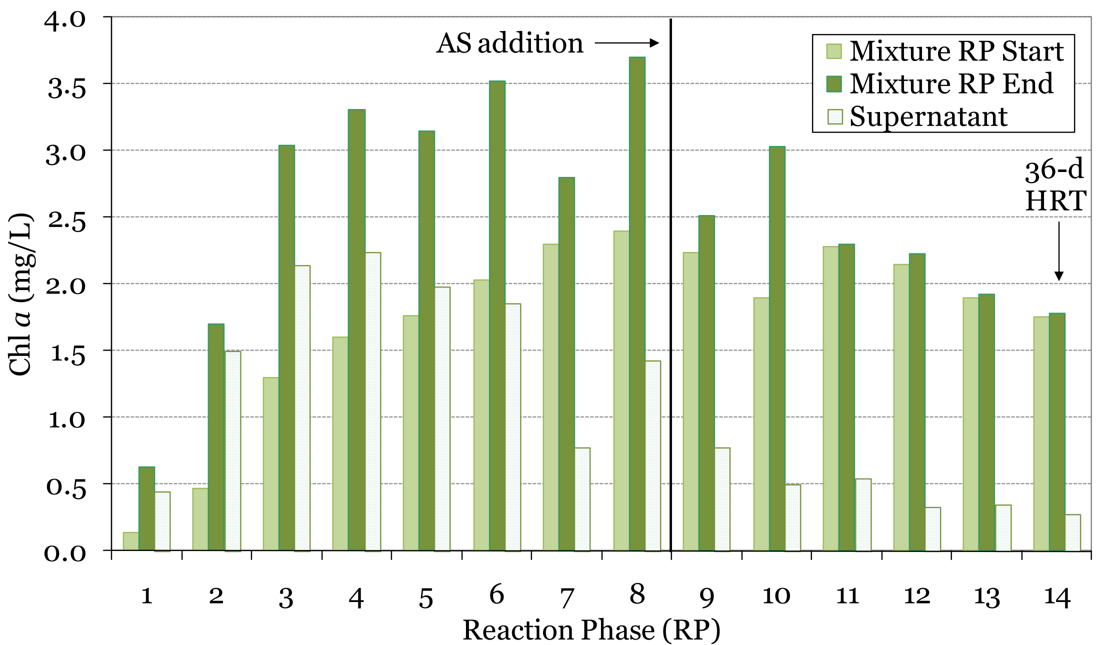

Figure 3-10. Chl *a* Concentrations at Start and End of Each RP.

(Note: results of replicates sampled at alternating RPs shown; each RP was 3 d except RP 14, which was 12 d.)

Natural sedimentation of suspended matter occurred in the SBRs during the 60-min settling periods at RP ends. A greater difference between mixture RP end and supernatant Chl *a* indicated greater microalgal settleability (Figure 3-10). Microalgae were growing rapidly during RPs 1 and 2, so they were unsettleable (i.e., showing similar supernatant and RP end concentrations for each RP). Microalgae exhibit a higher negative surface charge during exponential growth which keeps them separated by repulsive forces and prevents aggregation and settling (Becker 1994; Henderson et al. 2008). For example, Danquah et al. (2009) found that electronegativity of microalgal cells decreased from -43 mV during exponential growth to -35 mV during low growth and enabled greater agglomeration.

There was more pronounced difference between supernatant and RP end Chl *a* from RP 3 onwards (Figure 3-10) probably due to increased bioflocculation. Bioflocculation is generally defined as spontaneous clumping and settling of microalgae (Becker 1994; Richmond 2004), but it was easily induced in the SBRs once the growth rate slowed down. In support of this phenomenon, a significant, strong, negative correlation existed between microalgal settleability and growth rate (Figure 3-11). Settleability was calculated as the percent of Chl *a* settled from supernatant at RP end (Equation 2-5b), and growth rate was calculated according to Equation 3-2. (These time-weighted, average growth rates were not actually expected to be constant over the entire RP, but they were still informative for examining the settleability correlation.) The relationship was stronger with the exclusion of data from RPs 1 and 2 when microalgae were growing rapidly (r[14]=-0.71 vs. r[12]=-0.85). Lavoie and de la Noüe (1987) also showed that microalgal settleability increased with cell age due to increasing cell density (i.e., from 1.06-1.13 g/mL). Settleability within the SBRs certainly improved from 12 to 85% as cells aged and growth rate decreased (Figure 3-11). This trend indicated that maximum biomass production and maximum settleability may be mutually exclusive, and that harvesting via sedimentation may be more efficient for mature, dense cultures compared to young, unrestricted cultures. Therefore, subsequent experiments incorporated approximately 2 HRTs of acclimation time.

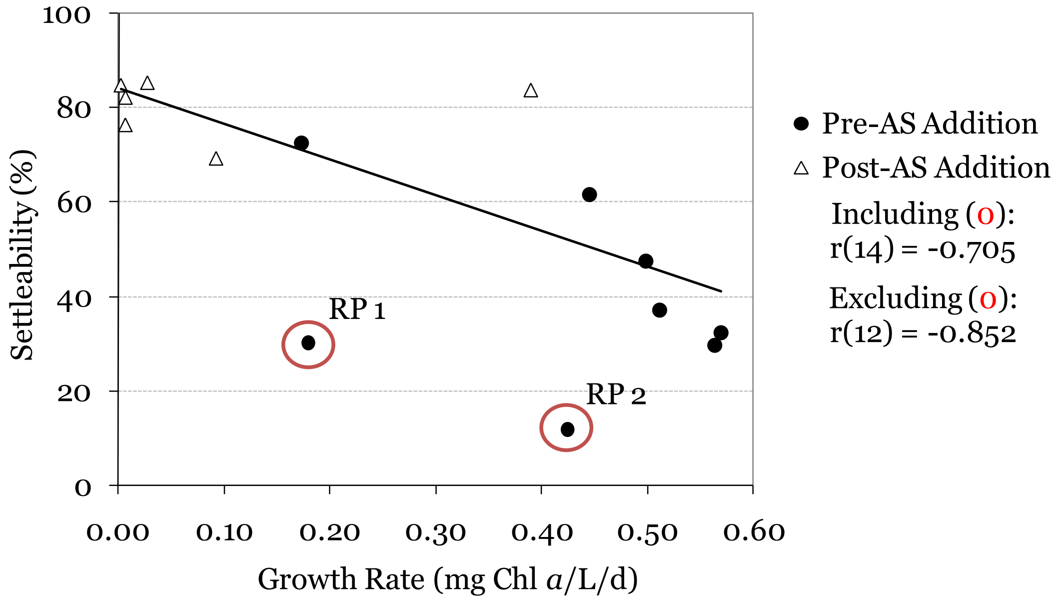

Figure 3-11. Effect of Microalgal Growth Rate on Settleability.

$$k = \frac{C_t - C_o}{t} \qquad \text{3-2}$$

Where: *k = growth rate (mg/L/d)*
C_t = Chl a concentration at time t *(mg/L)*
C_o = Chl a concentration at time 0 *(mg/L)*
t = time interval for one RP (d)

3.3.3.3 Supernatant Characteristics

In addition to Chl *a* (Section 3.3.3.2), COD, TSS, and turbidity of the supernatant were also influenced by biomass settleability, which has implications for wastewater treatment by microbial biomass. Generally, conventional AS treatment selects for the dominance of highly settleable flocs resulting in treated effluent supernatant (e.g., typically < 50 mg/L TSS and < 100 mg/L COD at CWTP [Table 2-1]). EBFs produced naturally by bacteria and microalgae likely enhanced floc formation and, thus, settleability of the biomass (Salehizadeh and Shojaosadati 2001) within the 1-h settling period of the RPs resulting in lower supernatant TSS and Chl *a*. Unsettleable solids were reduced from 112 to 53-68 mg/L TSS and suspended microalgae from 1.4 to 0.22-0.34 mg/L Chl *a* (Figure 3-12). Supernatant COD was more impacted by biomass settleability during RPs 1-8 because these conditions were conducive of aerobic bacterial growth. For RP 9, supernatant COD spiked due to solubilisation of AS resulting from anaerobic conditions and then decreased over time (Figure 3-12) from dilution by primary wastewater and oxidation. Turbidity, which can be used to approximate solids contents, was comparable in the supernatant irrespective of AS addition because of the high settleability of AS. Overall, AS addition added settleable solids to the culture while reducing supernatant solids (including microalgae), which thereby improved microbial settleability from 12-73% without AS to 62-85% with AS (Figure 3-11).

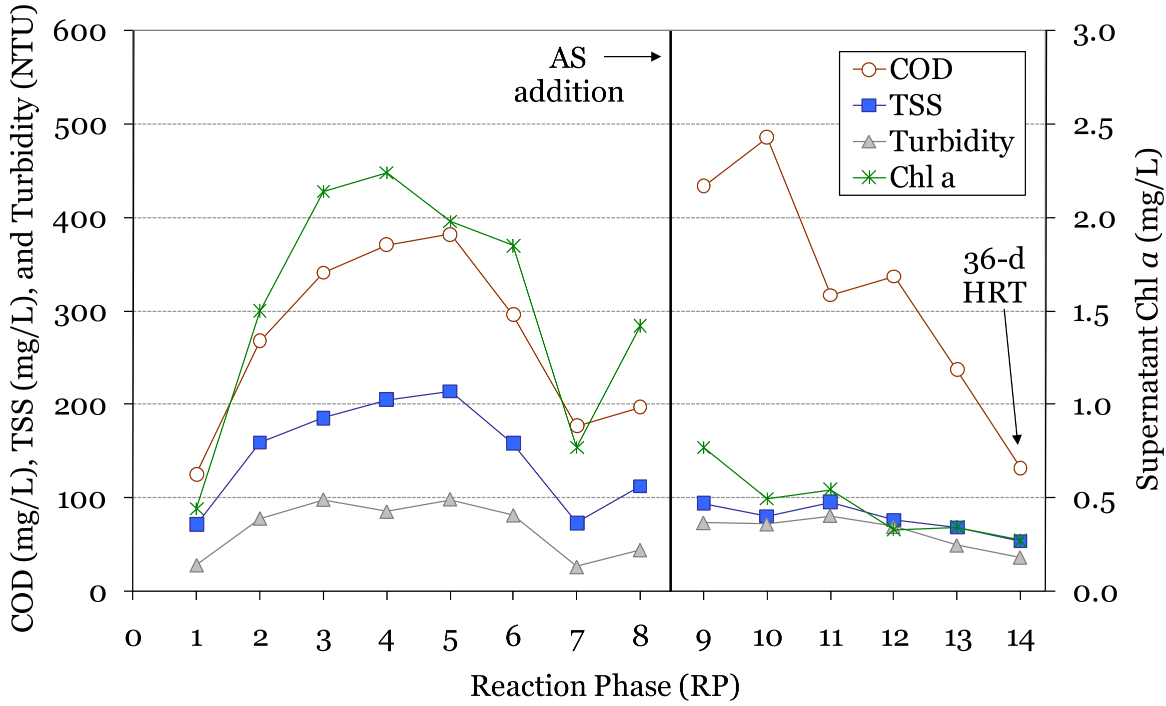

Figure 3-12. Characteristics of Supernatant at RP Ends.

(Note: results of replicate SBRs sampled at alternating RPs shown.)

3.3.4 Biomass Stabilisation Experiment

Following RP 14, some anaerobic microbial culture from SBR2 was diluted to determine the effect of reduced solids content with the desire to restore microbial growth and aerobiosis. Four, 2-L reactors were established (Section 2.2.1) and surrounded with tin foil to eliminate light penetration through the sides. Two reactors (replicates) received 1 L SBR2 culture and 1 L tap water (i.e., 50% dilution [D50]), and two reactors (replicates) received 0.5 L SBR2 culture and 1.5 L tap water (i.e., 75% dilution [D75]). Evaporative losses were regularly recovered with DI water to maintain a 2-L volume in each reactor.

The batch experiment was run for 12 d. TSS decreased while Chl *a* remained unchanged in all cultures. These results indicated that bacteria and other organic matter degraded while microalgae endured, and, thus, microalgal content of the biomass correspondingly increased by 40% for the D50 and 34% for D75 (Figure 3-13). Diurnal pH and DO patterns characteristic of microalgal cultures (Section 3.3.2.2) returned after about 4 and 6 d for D75 and D50, respectively. D50 had lower and less pronounced fluctuations in pH and DO (i.e., 7.2-8.0 SU and 0-7.2 mg/L) than D75 (i.e., 7.3-8.7 SU and 0-8.1 mg/L). These differences were presumably due to greater microalgal photosynthesis by D75 resulting from 1) reduced shading, 2) slightly higher initial microalgal content (i.e., 2.4 vs. 2.2 mg Chl *a*/g TSS on Day 1 [Figure 3-13]) likely due to heterogeneity of SBR2 culture used to seed the reactors, and 3) likely lower concentrations of any toxic contaminants (e.g., elevated ammonia [Section 3.3.2.1]) within D75). These results further implied that pH and DO levels and the overall aerobic balance of the cultures were highly sensitive to microalgae and bacteria contributions. Therefore, inocula of subsequent experiments were standardised to ensure a consistent ratio of P6 and AS microbes for effective comparison across treatments (Section 4.1.1).

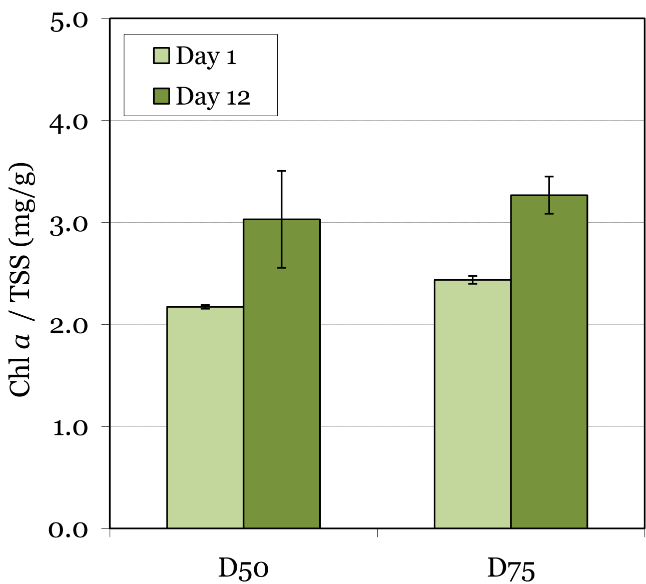

Figure 3-13. Chl *a* Contents of 50 and 75% Diluted SBR Cultures at Start and End of a 12-d Stabilisation Period (*n*=2).

3.3.5 Related Work

The experimental approach of this SBR experiment was similar to that of Gutzeit et al. (2005) who combined *Chlorella vulgaris* and 5% (v/v) AS from a WTP to form microbial (microalgal-bacterial) flocs. Feed water characteristics were similar between both studies. However, the lab-scale reactor operated by Gutzeit et al. (2005) had a much lower HRT of 1-3 d and received a smaller AS inoculum (i.e., 5 vs. 12% v/v) which would have diluted any excess solubilised sludge sooner and may have enabled faster microbial stabilisation. A much higher PAR of 2,000 $\mu mol/m^2/s$ in their study beget greater Chl *a* and DO (i.e., 15-37 mg/L and 0.2-1.5 mg/L respectively). In a larger, pilot-scale reactor with a 5- to 7-d HRT and 500 $\mu mol/m^2/s$, which are more similar to this study, supernatant contained 18 mg/L TSS and 0.22 mg/L Chl *a* (Gutzeit et al. 2005). These values are comparable to final supernatant concentrations reported herein (i.e., 68 mg/L TSS and 0.34 mg/L Chl *a* with 9-d HRT and 53 mg/L TSS and 0.27 mg /L Chl *a* with 36-d HRT). These findings support the well-accepted notion that PAR, HRT, and/or microbial species are determining factors in biomass productivity and concurrent wastewater treatment (Sheehan et al. 1998; Munoz and Guieysse 2006).

3.4 Summary

Glassware Experiments

- Wastewater from P6 had a greater quantity of diverse microalgae than P1, and removal of large microfauna (e.g., algivores) via coarse filtration facilitated greater microbial growth compared to unfiltered wastewater.
- AS was not successful as a rapid microalgal flocculant. However, longer mixing time of AS and P6 wastewaters enhanced biomass settleability.
- Microbial growth was influenced by the loading ratio of AS to P6 microalgae.

CSTR Experiment

- Replicate microbial cultures experienced natural divergence probably due to variation in the dynamic development of the microbial communities.
- Continuous reactors did not operate as intended. SBRs with larger, daily feeding and discharging steps (i.e., fed-batch design) were utilised for subsequent studies to allow deliberate biomass recycling.

SBR Experiment

- Microbial biomass grown in fed-batch SBRs on a mixture of oxidation pond and primary wastewaters from CWTP demonstrated wastewater treatment through ammonia reduction.
- As microalgae aged and culture concentration increased, decreasing growth rates positively impacted settleability via bioflocculation. This behaviour indicated that biomass productivity

may need to be sub-optimal in order to attain adequate settleability.

- Following AS addition, microalgae could not meet the oxygen demand, which caused a loss of biomass due to solubilisation of organic matter and/or aerobic microbial death. Wastewater treatment capacity and productivity decreased, but settleability increased.

The overall objectives of these preliminary experiments were to select appropriate inocula and laboratory setups, grow microbial flocs, and demonstrate wastewater treatment. Fundamental groundwork from these experiments provided a basis for designing more detailed experiments which could then be used to address more probing research questions. In summary, microbial symbiosis influenced growth, aerobiosis, and wastewater treatment capacity of the microbial cultures. Therefore, inoculum standardisation was initiated to regulate microalgal concentrations for subsequent experiments. Benefits of AS warranted future investigation, so approximately 2% AS inoculum was added to some reactors to encourage microbial symbiosis at a conservative level of TSS and to promote consistent microbial ratios. Subsequent experiments also incorporated an acclimation period to enhance biomass settleability prior to regular monitoring. SBR operation was chosen for future experimental setups to further examine settleable biomass recycling.

Chapter 4: Municipal Feed Water Selection Experiments Using Controlled Climate Laboratory SBRs

4.1 Experimental Design

Municipal wastewaters have been used for the culture of microalgae (Table 1-3), but those studies have not been conducted under the same conditions—ecological, climatic, and operational—so directly comparing site-specific studies can be of little value. It was unknown which municipal feed water would be optimal—secondary wastewater could enhance microalgal growth due to greater light penetration while primary wastewater could enhance bacterial growth due to greater C content (Table 2-1). Inoculating the cultures with AS was expected to further improve the settleability of microalgae and bacteria through co-flocculation since AS is widely used as a flocculating biomass in wastewater treatment (Forster and Water Environment Research Foundation 2003; Mara and Horan 2003; Alley 2007). Experiments were, therefore, designed to assess effects of feed water and climate on native biomass production, settleability, and composition since these factors influence the economics of microbial biofuel production. The overall objective was to ascertain which feed water may be optimal under typical climatic conditions for Christchurch, NZ in respect to these key economic pressures.

Four laboratory reactors were assembled and operated as SBRs (Section 2.2.2) under two controlled climates according to Table 4-1. Cold Studies (i.e., 410 µmol/m^2/s PAR for 9.6 h/d at 13 °C mean water temperature; n=2) and Warm Studies (i.e., 925 µmol/m^2/s PAR for 14.7 h/d at 21 °C mean water temperature; n=3) (Section 2.6.2) were conducted over a period of about six months in an enclosed laboratory room (337B) equipped with air temperature and humidity controls within UC's Civil and Natural Resources Engineering building. Irradiance cycles and SBR operation (e.g., fill, mix, waste; Appendix B) were automated through a computer (Section 2.2.2). Although three replicates were established for each climatic condition, one Cold Studies' replicate was later dismissed due to excessive operational downtime that could have biased results (Section 4.1.3).

4.1.1 Inoculum Standardisation

Microalgal communities in P6 wastewater used for inoculum varied over time (Appendix A) presumably in response to environmental conditions as was also observed by Novis (2007). In an effort to begin each replicate with relatively equal abundances of microbes adapted to the prescribed climatic conditions, P6 wastewater was 'standardised' immediately following sample collection. Inoculum was batch-grown in translucent, 30-L buckets under specific climatic conditions (Table 4-1) and mixed once daily. Incubation continued until P6 wastewater was standardised to 63 mg/L (as TSS) with 0.4 mg/L Chl *a*. If initial TSS was > 50 mg/L, such as during Christchurch summer (December-February), P6 wastewater was first diluted with tap

Table 4-1. Climatic, Startup, and Operational Conditions of Controlled Climate Experiments.

Parameter	Cold Studies (T2-3)[a]	Warm Studies (T4-6)
Climatic Conditions		
Daytime PAR (µmol/m²/s)	410	925
Day Length (h)	9.6	14.7
Water Temperature (°C)	13 ± 3	21 ± 3
Relative Humidity (%)	60	60
Initial Microbial Conditions		
P6 Inoculum (mg/L TSS)	63 ± 8	
P6 Inoculum (mg/L Chl *a*)	0.4 ± 0.1	
AS Inoculum for AP SBRs Only (mg TSS)	1,500	
Operational Conditions		
Operating Volume (L)[b]	21-24	
Water Depth (cm) [b]	30-34	
Mixing Speed (cm/s)	26	
Illuminated Surface Area (cm²)	707	
Reaction Phase (d)	1	
Hydraulic Retention Time (d)	8	
Solids Retention Time (d) [c]	8-40	

Note: *M* ± *SD* indicated where applicable; (a) T1 omitted due to downtime; (b) daily increase; (c) increase over time.

water to enable the inoculum to grow and adapt to the room's climate. This preliminary standardisation procedure minimised variation of the inoculum between replicates by pre-adapting the microbes to specific climatic conditions and adjusting the inoculum to a standard concentration while ensuring adequate TSS to prevent microbial washout. Standardisation periods for all P6 inocula ranged from 3 to 10 d (Appendix C).

4.1.2 Culture Acclimation and Operation

All SBRs were inoculated with 21 L standardised P6 wastewater containing 63 ± 8 mg/L TSS, and one SBR was also seeded with 1,500 mg (as TSS) or 2% v/v AS inoculum (AP) (Section 2.1.2). They were then continuously fed either tap water as a control (CO), secondary wastewater (SE), or primary wastewater (PE and AP). PE and AP were identical except only AP received the AS inoculum. Although continuously fed, supernatant was wasted only once daily, so SBR depth and volume increased over the course of each 1-d RP (Table 4-1). Mixture was only removed from the SBRs for sampling (i.e., about 2% per day or 3 L/week) at the end of light periods. This operational strategy maintained HRT and continually accumulated microbial biomass within the SBRs resulting in increasing SRT from 8-40 d over the monitoring period. In order to monitor more adapted, mature, and settleable cultures following SBR inoculation and feed initiation, microbial cultures were acclimated for 2 HRTs (i.e., 2 weeks), which agreed with the range of 5-14 d recommended for HRAPs by Al-Shayji et al. (1994). Then, biweekly sampling

was conducted for an additional 3 HRTs (i.e., 3 weeks) to elucidate trends of mature cultures and enable general comparison of treatments for design of subsequent studies.

4.1.3 *Difficulties Encountered*

Conclusions relating to the comparison of treatments were based on results from the second and third Cold Studies replicates only (T2 and T3) and all Warm Studies replicates (T4, T5, and T6). Random power interruptions and computer memory failures during the first Cold Studies replicate (T1) resulted in more than 50% downtime than other tests (Appendix C). As a consequence of these operational difficulties, T1 data were omitted from the following discussion and statistical analyses.

4.2 *Biomass Production*

4.2.1 *TSS Concentrations*

Since the SBRs were not operated at steady state, TSS (rather than productivity) was measured to provide a basis of comparison among the SBRs (Section 2.4.2). TSS of the wastewater cultures (SE, PE, and AP) increased over time reflecting microbial biomass growth, while TSS of CO decreased (i.e., from 63 on average at startup to < 30 mg/L) presumably due to lack of nutrients in tap water for growth and the ensuing microbial washout (Figure 4-1). TSS (and settleability) data were analysed using a linear mixed-effects model (LMM) (Section 2.7) to determine statistical differences between cultures (Table 4-2). TSS value and growth rate (i.e., evident from the linear trendlines as intercept and slope, respectively) were significantly different between all cultures except:

a) CO and SE were *only* different for intercept value (i.e., CO < SE) in the Warm Studies, and
b) PE and AP were *only* different for growth rate (i.e., PE < AP) in the Cold Studies.

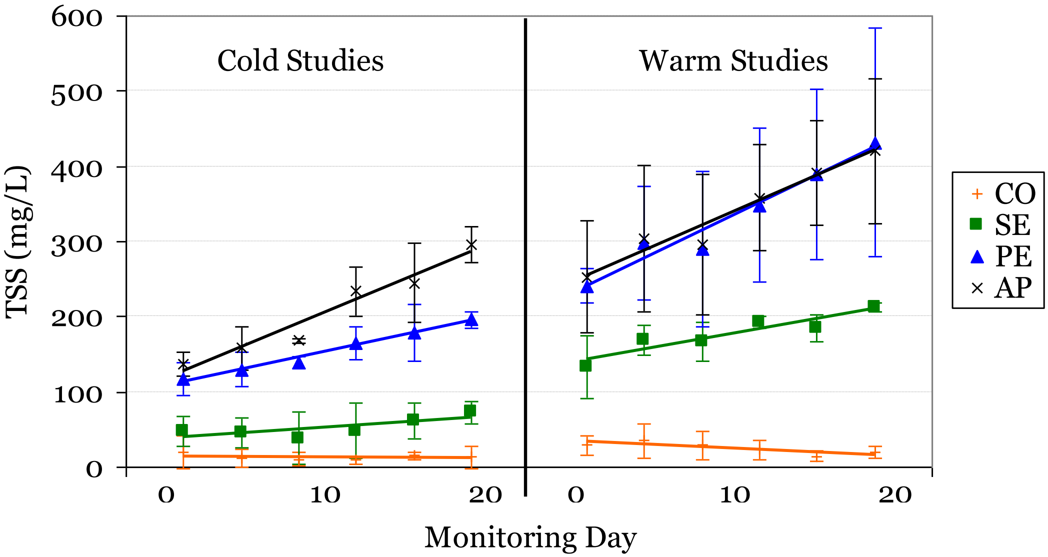

Figure 4-1. TSS Concentrations of SBR Mixtures over Time during Cold Studies (*n*=2) and Warm Studies (*n*=3).

Table 4-2. Summary of LMM Differences in TSS Intercepts/Slopes between SBRs for Climatic Conditions.

	Cold Studies			Warm Studies		
SBR	CO	SE	PE	CO	SE	PE
SE	ND/ND	-	-	Yes/ND	-	-
PE	Yes/Yes	Yes/Yes	-	Yes/Yes	Yes/Yes	-
AP	Yes/Yes	Yes/Yes	ND/Yes	Yes/Yes	Yes/Yes	ND/ND

Note: intercept ≈ value; slope ≈ rate of change; ND = no significant difference (i.e., $p > 0.05$); Yes = significant difference (i.e., $p < 0.05$).

However, CO and SE were *always* significantly different from PE and AP. These statistical outcomes (Table 4-2) likely occurred because of feed water characteristics—CO and SE both received low C loading while PE and AP both received higher C loading (i.e., $M \leq 90$ vs. 400 mg/L COD). Similarly, Kucnerowicz and Verstraete (1983) also found that AS-inoculated (AP) and non-inoculated cultures (PE) fed the same wastewater attained similar performance (e.g., TSS, nitrification) in the long run due to the ubiquitous nature of the microbes and the selective growth pressures of the feed water. Therefore, PE and AP data are typically lumped together in the following discussions in order to compare the effects of primary and secondary wastewaters (i.e., PE-AP vs. SE).

The principal differences in COD and TSS between the municipal feed waters (Table 2-1) were essentially due to their C contents because the solids were primarily organic (i.e., VSS/TSS ratio) (Scragg 2004). Most non-volatile solids (e.g., sand, grit) were removed upstream via screening and sedimentation, but the organics were degraded by the treatment process and eventually became assimilated into new microbial cells or enmeshed within the microbial flocs. COD loadings were 16.4 $g/m^2/d$ for primary wastewater to PE and AP and 3.7 $g/m^2/d$ for secondary wastewater to SE during this 8-d HRT experiment. Feed water effects (Figure 4-1) yielded final mean TSS:

a) 246 mg/L for PE-AP and 73 mg/L for SE for Cold Studies, and
b) 426 mg/L for PE-AP and 213 mg/L for SE for Warm Studies.

These values indicated about 240% (for Cold Studies) and 100% (for Warm Studies) greater biomass for PE-AP compared to SE at replicate ends (monitoring day 19). Therefore, influent C (and climate as discussed below) affected biomass production. Similarly, Cromar and Fallowfield (1997) achieved 159% greater TSS when increasing from 10 to 60 $g/m^2/d$ COD, and de Godos et al. (2009) observed 18% more biomass for an outdoor HRAP operated at 28.6 compared to 15.8 $g/m^2/d$ COD. Therefore, greater C loading afforded by primary wastewater grew higher TSS microbial cultures compared to secondary wastewater.

Climate also affected biomass production of the cultures (Figure 4-1). Initial TSS was higher under Warm Studies because the 2-HRT acclimation period (Section 4.1.2) facilitated greater

growth under the more favourable climate. In general, greater TSS continued to exist throughout the Warm Studies, indicating that Cold Studies' cultures were limited by temperature (i.e., 13 vs. 21 °C) and/or light (i.e., 30% of Warm Studies' radiation). Performance was improved for SE with significantly greater value and growth rate and also for PE with significantly greater growth rate during the Warm Studies compared to the Cold Studies (Table 4-3). At replicate ends (monitoring day 19), 193% (for SE) and 73% (for PE-AP) greater biomass was produced for Warm Studies compared to Cold Studies. Similarly, Voltolina et al. (2008) found that TSS of microalgal cultures increased by over 300% from winter to summer conditions at 11 °C higher temperature and 70% greater irradiance.

Table 4-3. Summary of LMM Comparison of Cold and Warm Studies for TSS and Settleability.

SBR	TSS		Settleability	
	Intercept	Slope	Intercept	Slope
CO	ND	ND	ND	Warm < Cold
SE	Warm > Cold	Warm > Cold	ND	ND
PE	ND	Warm > Cold	ND	ND
AP	ND	ND	ND	Warm > Cold

Note: intercept ≈ value; slope ≈ rate of change; ND = no significant difference (i.e., $p > 0.05$); differences significant from counterpart indicated (i.e., $p < 0.05$).

Variability existed between replicates, most notably for PE and AP during Warm Studies, as evident from the error bars (± 1 *SD*; Figure 4-1). Variability was attributed to natural divergence of the complex microbial communities (Matheson et al. 2008) and customary changes in the feed water over time depending on influence of industrial trade wastes, precipitation, and other factors at CWTP.

Irradiance heated the Cold Studies' cultures from about 8 to 18 °C over the 9.6-h light period and the Warm Studies' cultures from about 16 to 26 °C over the 14.7-h light period, which was a wider temperature range than is typical of outdoor HRAPs (e.g., 10 vs. ~5 °C). Low temperatures during the Cold Studies may have limited photosynthetic efficiency of microalgae even at lower irradiance compared to the Warm Studies by slowing CO_2 fixation and reducing protection and repair of photosystem II, which is required for the light reactions of photosynthesis (Richmond 2004). Vonshak et al. (2001) observed greater photoinhibition of microalgae (i.e., 0.66 vs. 0.70 efficiency) in outdoor PBRs following exposure to sub-optimal (morning) temperatures (i.e., 9 vs. 21 °C), indicating faster photosaturation (and reduced photosynthetic efficiency) of Cold Studies' cultures at their lower initial temperature. Productivity could potentially benefit with early morning heating as demonstrated up to 60% by Vonshak et al. (2001) by increasing the photosaturation threshold.

Mean temperature of primary wastewater at CWTP was about 17 °C (Table 2-1). Generally, bacterial activity nearly doubles for every 10 °C increase up to optimum temperature (Gerardi 2006). The daily heating probably greatly enhanced bacterial growth in the Cold Studies, but had less influence in the Warm Studies. However, Mayo and Noike (1996) grew cultures of heterotrophic bacteria and *Chlorella*, and they found no difference in bacterial densities grown within the range 10-20 °C at pH 7 SU under stable conditions. Nor was there any difference within the range 10-20 °C at pH 10 SU, but bacterial densities grown at pH 10 SU were greater than those grown at pH 7 SU as attributed to microalgae reducing bacterial growth by competition for glucose at neutral pH (Mayo and Noike 1996). Despite these disparities for bacterial growth, microalgae comprised a larger portion of the biomass (Section 6.2.2), so they were more of a determining factor in the overall biomass production.

4.2.2 pH and DO Concentrations

Primary wastewater inherently contained heterotrophic microbes and had greater oxygen demand where as secondary wastewater had most of the oxygen demand and heterotrophs removed by upstream treatment. Although both feed wastewaters had a pH of 7.4 SU, their different carbonaceous content (Table 2-1) influenced the pH and DO of the cultures, which likely affected the microbial growth responses. For example, PE and AP had lower pH (i.e., 7.5/8.5 and 7.2/8.0 SU for Cold/Warm Studies, respectively) probably due to greater respiration by bacteria and other heterotrophs within these cultures compared to SE (i.e., 8.3/10.2 SU for Cold/Warm Studies) (Table 4-4). In reality, bacterial growth may even have been inhibited in SE cultures at pH > 9 SU (Mara and Horan 2003). Additionally, pH also likely affected microalgal growth since more dissolved CO_2 was available for photosynthesis at lower pH due to carbonate chemistry (Liehr et al. 1988). However, Brune and Novak (1981) found that C-limited growth of microalgae was unaffected within the pH range of 7-10 SU.

Table 4-4. Summary of pH and DO Measurements of SBR Mixtures Recorded at 15-min Intervals for Cold and Warm Studies.

Parameter	Climate	Concentration (*M* ± *SD*)		
		SE	PE	AP
pH (SU)	Cold	8.3 ± 0.8	7.5 ± 0.7	7.2 ± 0.7
	Warm	10.2 ± 0.6	8.5 ± 1.0	8.0 ± 0.8
DO (mg/L)	Cold	9.8 ± 3.3	4.6 ± 4.0	2.9 ± 2.8
	Warm	12.5 ± 4.3	8.0 ± 4.8	5.6 ± 3.8

In all SBRs, microalgae supported daytime aerobic bacterial respiration and wastewater degradation to varying degrees without requiring mechanical aeration as evident by the mean DO levels (Table 4-4). However, photooxidative damage to microalgae can occur above 100% DO saturation (i.e., 8.9 and 10.5 mg/L at 21 and 13 °C, respectively) (Suh and Lee 2003). For instance, photosynthetic efficiency has been shown to decrease by 35% at 100% DO saturation

(Becker 1994). Becker (1994) recommended that oxygen levels not exceed 21% DO saturation (i.e., the oxygen content of air: 1.9 and 2.2 mg/L at 21 and 13 °C, respectively) to maintain photosynthetic efficiency. Meanwhile, the absence of malodours indicated that atmospheric oxygen absorption was adequate to sustain nighttime respiration, which depleted DO in the absence of photosynthesis (i.e., minimum 4 mg/L for SE and 0 mg/L for PE and AP [data not shown]). Consequently, primary wastewater seeded with AS provided the most amenable pH (i.e., M=7.2-8.0 SU) and DO (i.e., M=2.9-5.6 mg/L) for microbial growth compared to other cultures during both climatic conditions (Table 4-4).

4.3 Biomass Settleability

Settleability was quantified using Imhoff Cones (Figure 4-2) to assess culture harvestability via natural sedimentation of suspended solids. Sedimentation occurred daily in the SBRs during the darkened 60-min settling periods, and it was controlled by two mechanisms: bioflocculation and stable floc formation. Bioflocculation is clumping and settling of microbes (Becker 1994; Richmond 2004) that was induced when culture mixing ceased. Stable, compact flocs also developed as a result of various growth conditions such as nutrient loading, DO, and SRT (Liu and Tay 2004; Adav et al. 2008).

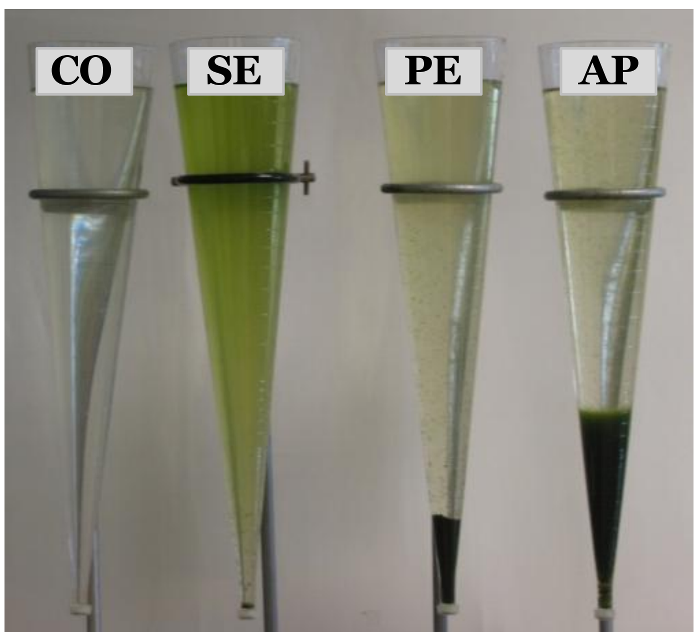

Figure 4-2. Dark-Induced Sedimentation Typical of Microbial Cultures after 60 min in Imhoff Cones during Cold Studies.

Generally, settleability was not significantly affected by climate (Figure 4-3a; Table 4-3), but it was affected by wastewater feed (Figure 4-3b; Table 4-5)—being much greater for PE-AP (i.e., M=76, SD=21%) than SE (i.e., M=22, SD=22%) cultures. Patterns of statistical significance for settleability (Table 4-5) were similar to those for TSS production (Table 4-2) probably because

settleability was calculated from TSS (Section 2.4.3). Cultures were significantly different for value and slope except:

a) CO and SE were *only* different for slope in the Warm Studies (i.e., CO < SE),
b) AP was *only* different from PE for slope (i.e., AP < PE) and value (i.e., AP > PE) in the Cold Studies, and
c) AP was not significantly different from CO and SE for slope in the Cold Studies.

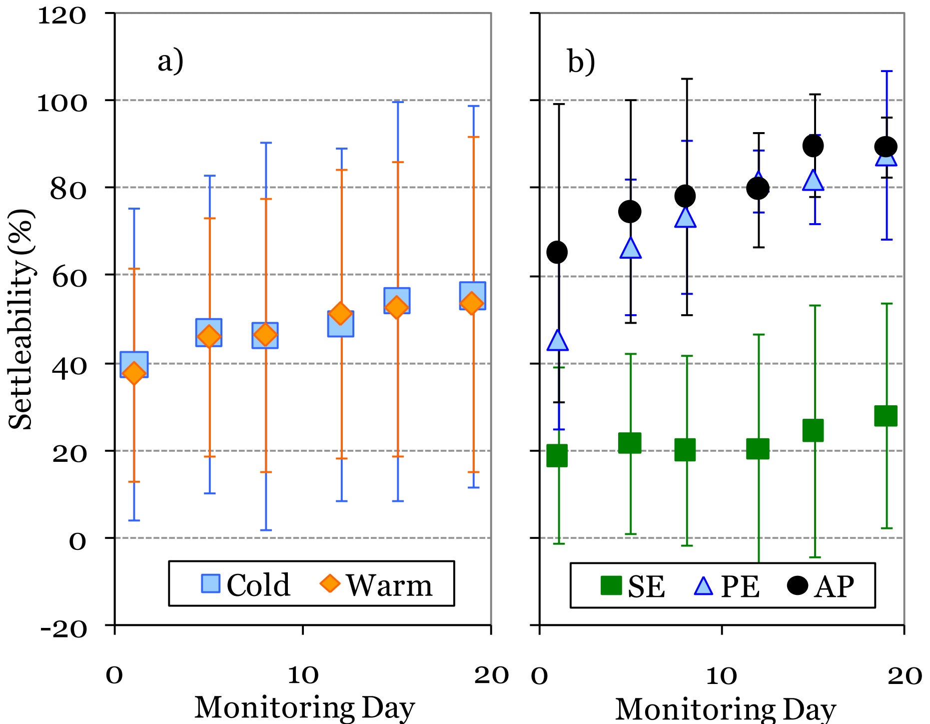

Figure 4-3. Settleability of Microbial Cultures over Time by a) Cold (*n*=2) and Warm (*n*=3) Climates and b) SBR Mixture (*n*=5).

Table 4-5. Summary of LMM Differences in Settleability Intercepts/Slopes between SBRs for Climatic Conditions.

SBR	Cold Studies			Warm Studies		
	CO	SE	PE	CO	SE	PE
SE	ND/ND	-	-	ND/Yes	-	-
PE	Yes/Yes	Yes/Yes	-	Yes/Yes	Yes/Yes	-
AP	Yes/ND	Yes/ND	Yes/Yes	Yes/Yes	Yes/Yes	ND/ND

Note: intercept ≈ value; slope ≈ rate of change; ND = no significant difference (i.e., $p > 0.05$); Yes = significant difference (i.e., $p < 0.05$).

Top settleability was achieved using AP (Figure 4-3b), but it was only statistically greater than PE for Cold Studies ($t[39]=13.27$, $p < 0.001$; Appendix C). This result was somewhat unexpected since AP was growing at a significantly greater rate than other cultures during the Cold Studies ($t[39]=-6.86$ to -3.31, $p \leq 0.001$ to 0.002; Figure 4-1), which was previously associated with

decreased settleability via bioflocculation (Section 3.3.3.2). To explain this, stable floc formation likely had a greater impact on settleability than bioflocculation in AP due to strong coagulation with AS. Cursory microscopic observations confirmed that P6 inocula consisting predominantly of dispersed microbes (i.e., < 100 μm) developed over the duration of the experiments into flocs ≤ 1,000 μm in AP compared to ≤ 500 μm in SE. Gutzeit et al. (2005) reported that microbial (microalgal-bacterial) flocs fed with primary wastewater were predominantly 400-800 μm, which were comparable to PE in this study. Liu and Tay (2004) also found that larger flocs formed at greater organic loading, so this would have enhanced settleability of larger primary wastewater flocs over time compared to smaller secondary wastewater flocs since settling velocity is proportional to particle size (and density) according to Stokes' Law (Batchelor 1967). Near the end of the experiment, rapid sedimentation of larger flocs was observed (Figure 4-4), which suggested that sedimentation time may be reduced (as investigated in Section 5.4.2).

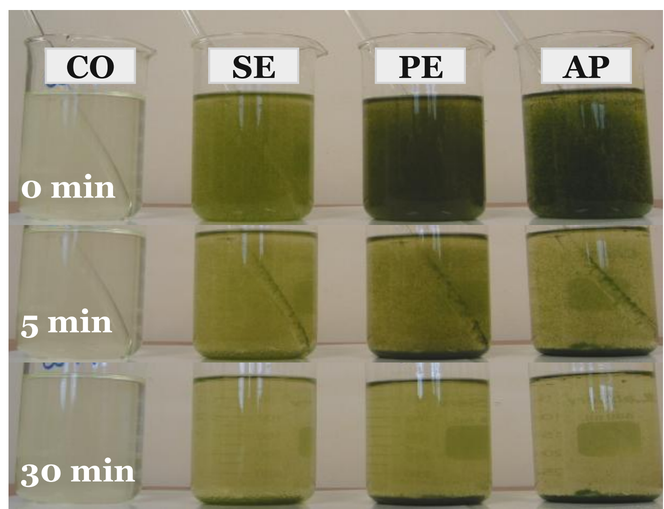

Figure 4-4. Sedimentation of Microbial Cultures after 0, 5, and 30 min.

The effect of light on sedimentation was also investigated. At the end of the light period and also at the start of the dark period, SV was measured (Figure 4-5). The 60-min SV was significantly greater in the dark compared to the light ($t[115]=2.90$, $p=0.005$). Other researchers have also demonstrated reduced sedimentation of microbial cultures in light (Lavoie and de la Noüe 1987; Danquah et al. 2009). Lavoie and de la Noüe (1987) stated that light-induced photosynthesis caused thermal convection currents and some cell flotation to occur (i.e., from micro-O_2 bubbles similarly to DAF effects) whereas Danquah et al. (2009) stated that greater electronegativity of actively photosynthesising cells decreased bioflocculation. During illuminated sedimentation of these cultures, both periodic autoflotation and decreased bioflocculation of flocs were observed to reduce settleability, which agreed with the previous studies. Interestingly, autoflotation and/or biomass bulking (i.e., resistance of bioflocculated sludge to settle) only occurred for PE and AP and only during the Warm Studies (Figure 4-6) possibly because cells were more densely concentrated and more active at higher temperature. The sedimentation impedances ceased

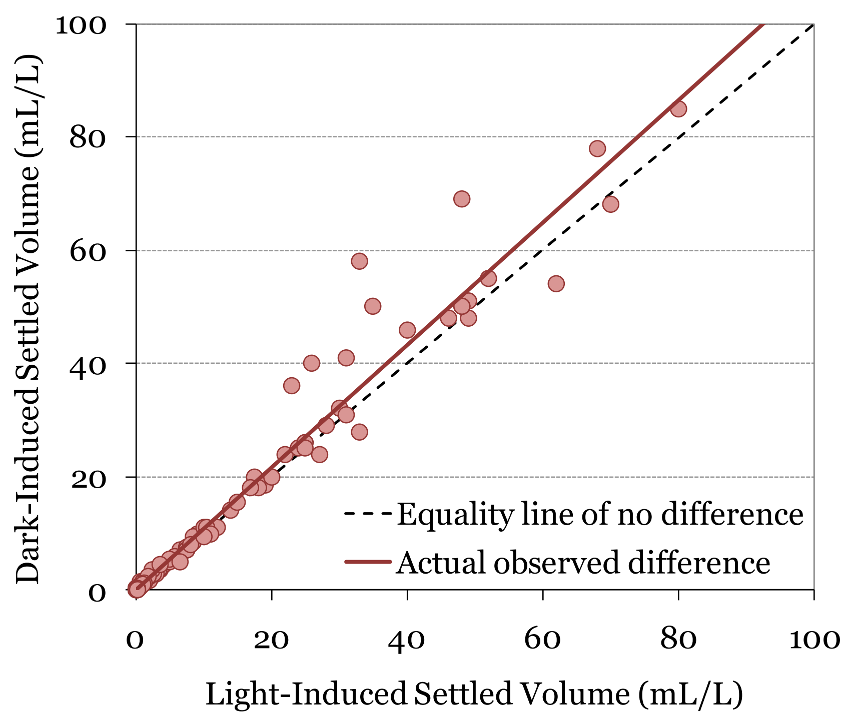

Figure 4-5. Effect of Light on 60-min Settled Volumes in Imhoff Cones.
(Note: solid line indicated greater sedimentation occurred in darkness than light.)

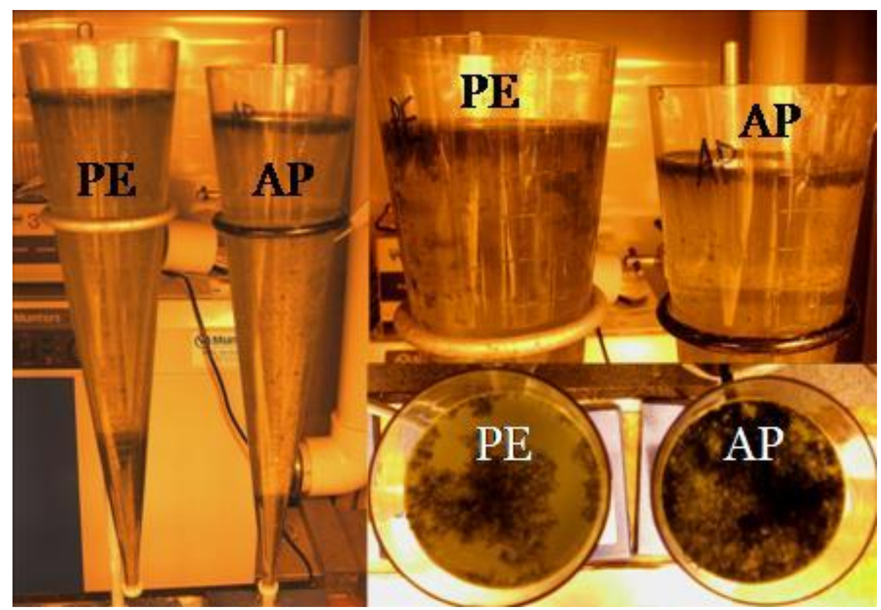

Figure 4-6. Periodic Light-Induced Autoflotation of Microbial Flocs in Primary Wastewater Cultures to Top of Imhoff Cones during Warm Studies' Sedimentation.

before measuring the 60-min SV except that autoflotation periodically persisted beyond 60 min of sedimentation when illuminated. These findings suggest that large-scale sedimentation of cultures should be conducted in darkness (e.g., at night or in opaque settling cones) for greatest harvesting efficiency, so all cultures continued to be settled exclusively in darkness.

4.4 *Summary*

- Microbial growth in Cold Studies was limited by lower light and/or temperature. Productivity was 73-193% greater in Warm Studies, confirming that microbial growth was highly sensitive to seasonal variations, which full-scale applications need to thoroughly consider when planning for annual productivities.
- Primary wastewater was a better substrate for growing microbial biomass compared to secondary wastewater on account of greater C content.
- Secondary wastewater was not suitable for microbial growth most likely because of insufficient dissolved CO_2, bacterial inhibition at elevated pH, and reduced photosynthetic efficiency of microalgae at supersaturated DO levels.
- Photosynthetic oxygenation of wastewater by microalgae kept all cultures aerobic. This advantage supports the shift towards more sustainable wastewater engineering alternatives that rely on biological processes.
- Biomass settleability was governed by microbial aggregation into stable, compact flocs as cultures aged and daily-induced bioflocculation during settling periods. Larger floc sizes were measured in primary wastewater cultures which consistently demonstrated greater settleability compared to secondary wastewater cultures.
- Sedimentation in darkness was recognised to improve experimental design since it resulted in greater biomass settleability than sedimentation in light.
- A mixture of primary wastewater, AS, and indigenous microalgae produced the greatest biomass and settleability when grown in laboratory SBRs under warm and cold climates.

These experiments contributed to understanding the effects of selected climatic conditions and feed wastewaters on production and settleability of microbial biomass. The benefits of increased C loading afforded to microbial cultures by primary wastewater and AS despite reduced light penetration were demonstrated. Utilisation of these less-treated wastewaters for microbial biomass production could result in reduced cost to WTPs compared to secondary wastewater cultures since energy requirements intensify with every level of treatment.

Chapter 5: Retention Time Experiments Using Controlled Climate Laboratory SBRs

5.1 *Experimental Design*

Experiments detailed in Chapter 4 demonstrated the benefits of using AS inoculum with primary wastewater feed for microbial biomass production. However, results indicated that these cultures could still be C limited. Shortened retention times could theoretically increase productivity, but would settleability be compromised? To address these questions, effects of SRT and HRT on biomass productivity and settleability were investigated. It was expected that as retention times decreased, productivity would increase due to greater nutrient loading, and that settleability would decrease due to faster microbial growth.

Three laboratory SBRs (Section 2.2.2) were arranged (Figure 5-1) and operated constantly at three different SRTs while sequentially being subjected to different HRTs (Table 5-1). The SBRs were evaluated using warm climatic conditions only since less operational difficulties were encountered with these conditions and because cultures were more growth limited in a colder climate. Two replicate experiments (T1 and T2) were conducted in an enclosed room (337B) equipped with air temperature and humidity controls within UC's Civil and Natural Resources Engineering building.

Figure 5-1. Tri-SBR Setup (Left) and Data Logging and Control Interface (Right) for Retention Time Experiments within Controlled Climate Laboratory Room.

Table 5-1. Startup, Operational, and Climatic Conditions of Retention Time Experiments.

Parameter	Value
Initial Microbial Conditions	
P6 Inoculum (mg/L TSS)	67 ± 0.8
P6 Inoculum (mg/L Chl *a*)	0.4 ± 0.0
AS Inoculum (mg TSS)	1,600
Operational Conditions	
Operating Volume (L)	22.5
Water Depth (cm)	32.5
Mixing Speed (cm/s)	26
Illuminated Surface Area (cm^2)	707
Reaction Phase (d)	1
Hydraulic Retention Time (d)	1.4 ± 0.0 (H1.4), 2.0 ± 0.3 (H2), or 4.0 ± 0.3 (H4)
Solids Retention Time (d)	4.0 ± 0.1 (S4), 8.3 ± 0.8 (S8), or 12.0 ± 2.1 (S12)
Warm Climatic Conditions	
Daytime PAR (μmol/m^2/s)	925
Day Length (h)	14.7
Water Temperature (°C)	19 ± 3
Relative Humidity (%)	60

Note: *M* ± *SD* indicated where applicable.

5.1.1 *Inoculum Standardisation*

The P6 wastewater inoculum was standardised according to Section 4.1.1. Standardised TSS was 67 mg/L on average, which corresponded to 0.4 mg/L Chl *a*.

5.1.2 *Culture Acclimation and Operation*

SBRs were inoculated with 22.5 L standardised P6 wastewater and 1,600 mg (as TSS) or 2% v/v AS.. Continuous (drip) feeding (per Section 4.1.2) was not practical for these experiments since culture depth would have been widely variable to facilitate shorter HRTs, so primary wastewater was fed once daily to achieve a 4-d HRT. Microbial biomass was accumulated within the SBRs during each 1-d RP by wasting supernatant only, which maintained HRT and prevented washout during culture acclimation. Meanwhile, minimal volumes of mixture were sampled from the SBRs for monitoring purposes (i.e., 1 L/week) at the end of dark periods. Eckenfelder et al. (1992) stated that batch acclimation of AS could require anywhere from 0 to 6 weeks, and Al-Shayji et al. (1994) recommended 5-14 d for this using HRAPs. Within 2 weeks, all cultures were sufficiently dense (*M*=300, *SD*=19 mg/L TSS) and settleable (*M*=66, *SD*=4%) to avoid microbial washout. At this time, "regular" operation began and mixture (in lieu of a portion of supernatant) wastage was initiated at the end of dark periods to target specific SRTs. The cultures were monitored until a stable condition was reached for each HRT period—signified by ≤ 10% *SD* of TSS from three or more mixture samples collected over several days.

5.1.2.1 Retention Times

Generally, in wastewater treatment processes, HRT controls nutrient loading since it reflects influent flowrate, and SRT controls microbial growth rate since it reflects solids wastage and food (i.e., nutrients) to microorganism (F/M) ratio (Gray 2004). Biomass recycling achieves SRT greater than HRT since solids are kept in the culture longer than liquids. Previous cultures were C limited at 8-d HRT and 8-40 d SRT (Chapter 4), so shorter (4-, 2-, and 1.4-d) HRTs were examined at different yet constant (4-, 8-, and 12-d) SRTs (abbreviated S4, S8, and S12, respectively) in dedicated SBRs to elucidate effects of these retention times. A shorter HRT at a given SRT was expected to increase total TSS productivity (provided adequate light penetration for photosynthesis occurred) since greater nutrient loading could support greater cell densities, and a longer SRT achieved via biomass recycling was expected to increase settleability since microalgal growth rate and settleability were inversely correlated (Section 3.3.3.2). However, long SRT could also reduce microalgal productivity by reduced light penetration at higher TSS.

Solids recycling of 75-92% was achieved using 4- to 12-d SRTs (i.e., 1-1/SRT wasted). This range was close to the 3-10 d range recommended by Orhon et al. (2009) for good settleability of AS treating municipal sewage. SRTs were controlled by discharging equal total volume, but different ratios of supernatant and mixture, and then internally recycling settled solids (Section 2.2.2). Initially, mixture (Q_m) and supernatant (Q_s) wastages to maintain SRT were based on an expected settleability of 80% since this was the average settleability over time for AP reactors (Chapter 4). However, settleability was not consistent, so SRT was then more strictly controlled by measuring actual settleability prior to discharge. Exact wastages were then determined according to models (Figure 5-2) that simultaneously solved Equations 2-1 (for HRT) and 2-2

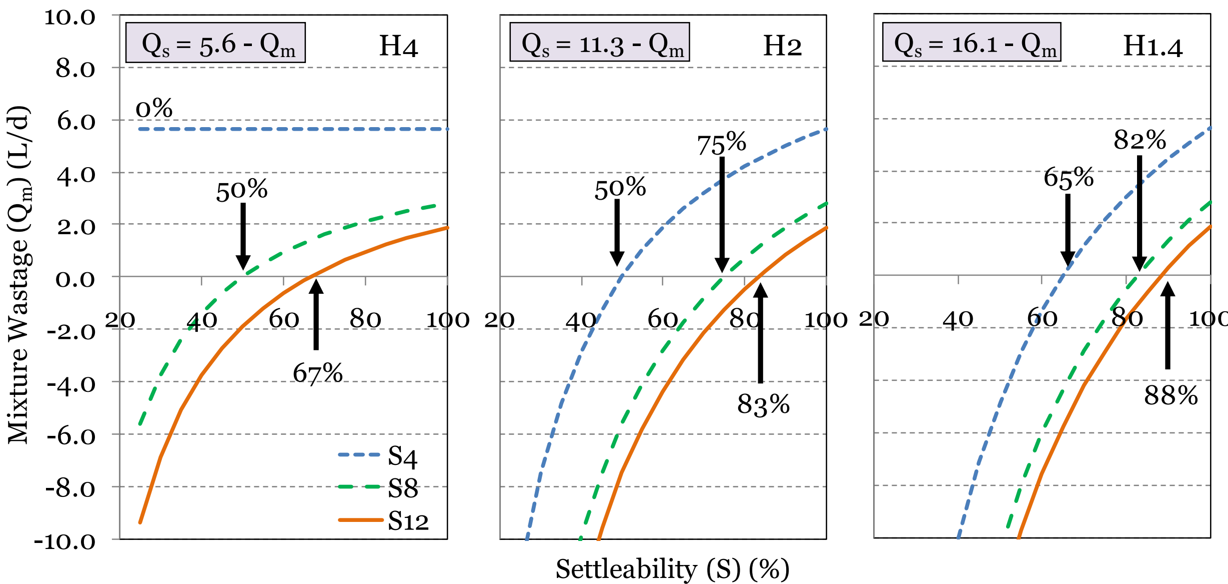

Figure 5-2. Mixture (Q_m) and Supernatant (Q_s) Wastage Models for SRT Control Based on HRT and Settleability. (Note: washout threshold indicated for minimum settleability [%] at Q_m=0.)

(for SRT) to maintain constant retention times within the SBRs. For example, to maintain 4-d HRT and 12-d SRT for a measured settleability of 80% in S12, 80% settleability is first located on the x-axis of the H4 wastage model (Figure 5-2). The point at which the mixture wastage line for S12 intersects with 80% settleability reveals that 0.9 L/d is required for Q_m on the y-axis. Since a total flowrate of 5.6 L/d is required to maintain 4-d HRT in the 22.5-L SBRs, Q_s is lastly calculated as 4.7 L/d by difference.

SRTs were initially evaluated at an average 4.0-d HRT in replicate (n=2). During this time, S4 essentially operated without cell recycle since SRT equalled HRT (i.e., only mixture was wasted). Comparison between replicates T1 and T2 indicated reproducible trends, so after achieving a stable condition for T2 only, HRT was sequentially shortened to 2.0 and then to 1.4 d on average by increasing total volumetric discharge. Meanwhile, SRTs were kept constant in all SBRs as summarised in Table 5-2 except for S12 while operated at a 1.4-d HRT due to solids washout occurring at this highest discharge volume (Section 5.4). S8 was not evaluated at 1.4-d HRT due to the greater wastewater requirements at this higher flowrate, and since it was not an extreme.

Table 5-2. Actual SRTs and HRTs of Microbial SBRs.

SBR / Nominal Retention Time	Actual SRT (d)		
	4.0-d HRT (n=45)	2.0-d HRT (n=16)	1.4-d HRT (n=10)
	M ± SD	M ± SD	M ± SD
S12	12.8 ± 1.6	11.8 ± 0.3	10.3 ± 2.8
S8	8.3 ± 0.8	8.0 ± 0.2	–
S4	4.0 ± 0.1	4.0 ± 0.1	4.0 ± 0.1

Note: only 4-d HRT conducted with two replicates.

5.1.2.2 Influent COD

Total COD of influent primary wastewater ($tCOD_i$) consists of inert (9%) and biodegradable (91%) fractions (Figure 5-3) (Orhon et al. 2009). Soluble COD of influent primary wastewater ($sCOD_i$) was approximately 50% of $tCOD_i$ (Table 2-1), and it impacted mixture TSS as previously discussed (Section 4.2.1). Therefore, $sCOD_i$ was measured regularly since it was anticipated to influence optimal retention times, and because it degraded over time (Appendix B). Unsurprisingly, spikes and dips of $sCOD_i$ appeared to affect corresponding changes in mixture TSS (Figure 5-4). Trends were not as apparent for 2- and 1.4-d HRTs due (not shown) to larger discharge volumes having greater impact on mixture TSS stability compared to $sCOD_i$ (Appendix C). Data collected during monitoring days 56-68 for T1 were omitted from the data analysis since $sCOD_i$ more than doubled during this period due to an operational issue at CWTP. Because of this variation (coupled to long-term wastewater data delays from ongoing database issues at CWTP), switching to artificial wastewater was considered since more consistent performance of the cultures could occur from uniform feed water quality (i.e., no microbial or nutritional variation). However, this path was not pursued since it would reduce compatibility of the studies

with previous and real-world (genuine wastewater) scenarios due to influence by microbes, colour, and other variables. Therefore, $sCOD_i$ unavoidably ranged from 63 to 296 mg/L and means were not consistent during different HRT periods (Table 5-3), which presented some unavoidable challenges in separating effects of HRT and $sCOD_i$ on the SBRs' productivity.

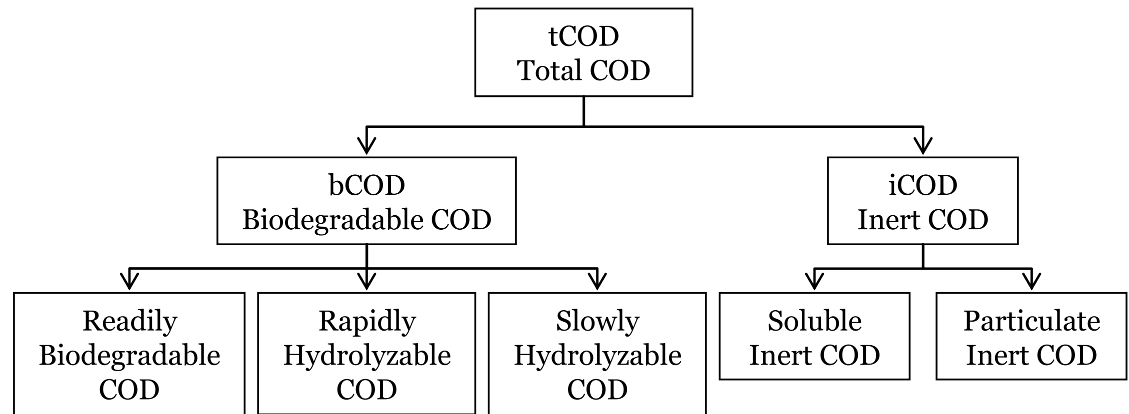

Figure 5-3. Major COD Fractions in Municipal Wastewater.

(Note: adapted from Orhon et al. [2009].)

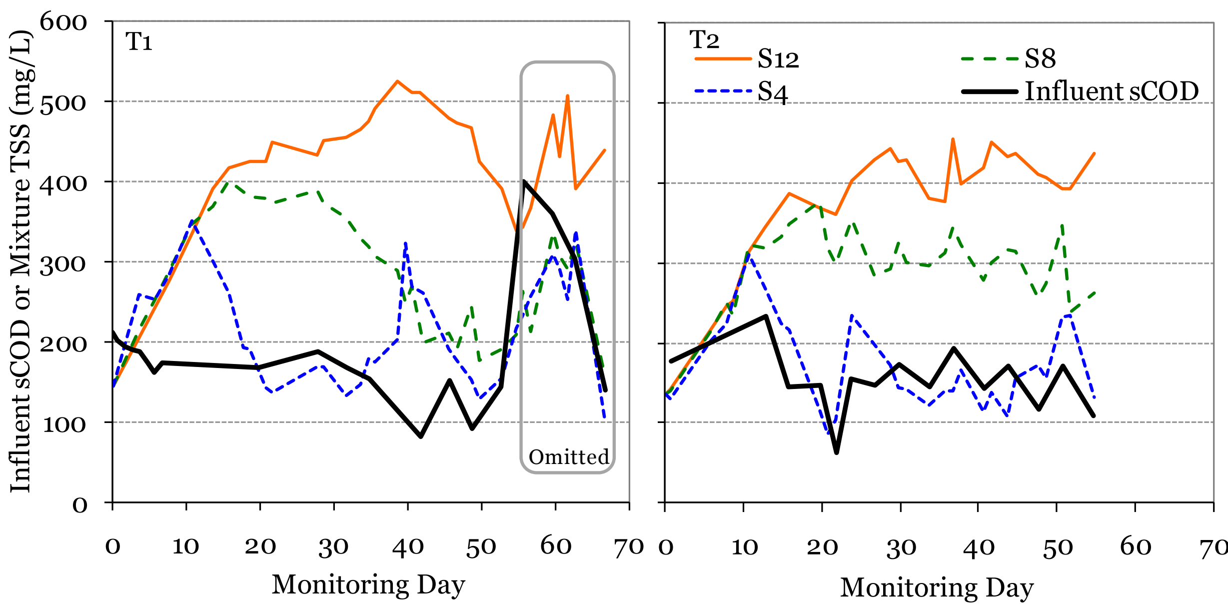

Figure 5-4. Influence of Influent sCOD on Mixture TSS of Replicate Microbial SBRs Operated at 4-d HRT.

Table 5-3. Influent tCOD and sCOD for SBRs According to HRT Period.

Period	$tCOD_i$ (mg/L)			$sCOD_i$ (mg/L)			$sCOD_i$ (g/m²/d)		
	M	±	*SD*	*M*	±	*SD*	*M*	±	*SD*
4-d HRT	337	±	83	159	±	38	12.7	±	3.0
2-d HRT	460	±	106	193	±	55	30.7	±	8.7
1.4-d HRT	267	±	85	141	±	44	32.0	±	10.0
All	360	±	119	173	±	63		-	

5.2 *Biomass Production*

Performance of the SBRs (e.g., productivity, settleability, etc.) fluctuated during each HRT period despite consistent operational and climatic conditions. These fluctuations were most likely due to microbial response to variations in the primary wastewater (e.g., $sCOD_i$ [Section 5.1.2.2]). Hence, it was impossible to reach an absolute steady state for the cultures, but overriding trends were still evident as discussed below.

5.2.1 *Total Productivity*

Biomass production was affected by how much biomass (TSS) was in the system and how fast it grew. SRT effectively controlled the mean microbial growth rate (μ) through solids recycling/wastage (i.e., 1/SRT=0.08, 0.12, and 0.25 d^{-1} for S12, S8, and S4, respectively). Total TSS productivity ($g/m^2/d$) was calculated from flowrates and solids' contents of discharged supernatant and mixture of cultures; illuminated surface area of the SBRs (i.e., 707 cm^2) was also incorporated (Equation 2-4). Total TSS productivities (and other critical measurements) are summarised in Table 5-4. Because the SBRs were operated over long-term under stable conditions (except S12 during 1.4-d HRT) while discharging different culture fractions (Section 5.1.2.1), total productivity was used (rather than simply mixture TSS as per Chapters 3 and 4) to compare solids production among the SBRs and across different studies.

Total productivities ranged from 7.7 to 24.3 $g/m^2/d$ during 4-d HRT and averaged 10.7, 11.7, and 14.2 $g/m^2/d$ for S12, S8, and S4, respectively (Table 5-4). These values agreed with those in the literature for outdoor systems dominated by green microalgae ranging from 10-35 $g/m^2/d$ (Becker 1994; Heubeck and Craggs 2007). Mean and maximum TSS productivities were expectedly less than those obtained in outdoor HRAPs operated at 4-d HRT with CO_2 addition (i.e., 20.7 and 30.8 $g/m^2/d$) (Park and Craggs 2010). S4 had greatest productivity despite lowest TSS because of its high growth rate. Throughout the experiment, S4 productivity was the most variable due to least biomass recycling while S12 was generally the most stable (Figure 5-5 and evident from Table 5-4 *SD* values) probably because S4 was comprised of younger and less biomass that was more impacted by changes in feed water compared to older, more robust S12 biomass (Figure 5-4).

HRAPs (and comparable systems to them) are rarely operated at < 4-d HRT due to washout concerns. However, maximum productivity was sought, and biomass recycling (i.e., SRT > HRT) reduced the likelihood of washout since microbes could grow slower at longer SRT. During 2-d HRT, mean total productivity for S12 doubled to 21.6 $g/m^2/d$, increased for S8 by 58% to 18.5 $g/m^2/d$, and increased for S4 by 38% to 19.6 $g/m^2/d$ (Table 5-4). The total productivity range of 13-29 $g/m^2/d$ TSS was comparable to that observed by Eisenberg (1981) operating outdoor HRAPs at 2-d HRT (i.e., 21-31 $g/m^2/d$ VSS). Greater nutrient loading to all cultures at this

Table 5-4. Critical Parameters of Microbial SBRs Operated at Different SRTs and HRTs.

Parameter	SBR	*n*	Minimum	Maximum	*M*	±	*SD*
4-d HRT							
Mixture TSS (mg/L)	S12	45	339	525	427	±	46
	S8	45	177	400	303	±	58
	S4	46	86	323	178	±	54
Total Productivity ($g/m^2/d$)	S12	38	7.8	13.6	10.7	±	1.5
	S8	38	7.7	16.4	11.7	±	2.1
	S4	37	8.2	24.3	14.2	±	4.0
Settleability (%)	S12	38	73	97	87	±	7
	S8	38	68	95	82	±	7
	S4	37	36	91	69	±	13
Settleable Productivity ($g/m^2/d$)	S12	38	5.6	11.1	9.3	±	1.3
	S8	38	6.0	12.8	9.6	±	1.8
	S4	37	4.2	17.5	9.9	±	3.3
2-d HRT							
Mixture TSS (mg/L)	S12	16	652	871	794	±	65
	S8	17	357	560	463	±	66
	S4	16	204	407	254	±	53
Total Productivity ($g/m^2/d$)	S12	16	17.0	26.3	21.6	±	2.5
	S8	17	13.4	24.2	18.5	±	3.1
	S4	15	16.0	29.0	19.6	±	3.0
Settleability (%)	S12	16	93	98	95	±	2
	S8	17	85	92	89	±	2
	S4	16	71	82	76	±	3
Settleable Productivity ($g/m^2/d$)	S12	16	16.4	25.2	20.6	±	2.4
	S8	17	12.0	20.5	16.5	±	2.7
	S4	15	12.0	21.2	14.9	±	2.2
1.4-d HRT							
Mixture TSS (mg/L)	S12*	13	487	766	612	±	100
	S4	10	196	295	246	±	35
Total Productivity ($g/m^2/d$)	S12*	13	13.1	30.7	18.0	±	4.7
	S4	10	14.3	31.2	19.5	±	5.2
Settleability (%)	S12*	13	73	97	91	±	7
	S4	10	73	91	83	±	5
Settleable Productivity ($g/m^2/d$)	S12*	13	12.1	22.5	16.2	±	3.2
	S4	10	12.1	22.7	16.1	±	3.3

*SBR was not able to be held at constant SRT during this period.

shorter 2-d HRT (i.e., 30.7 vs. 12.7 $g/m^2/d$ $sCOD_i$ for 4-d HRT [Table 5-3]) sustained greater biomass (TSS) and demonstrated the value of biomass recycling via higher SRT to obtain greater productivity. Others also found that microbial productivity of HRAPs increased at higher COD loading (Cromar and Fallowfield 1997; de Godos et al. 2009). Productivity was now greatest for S12 since its increased TSS overcompensated for its slower growth rate.

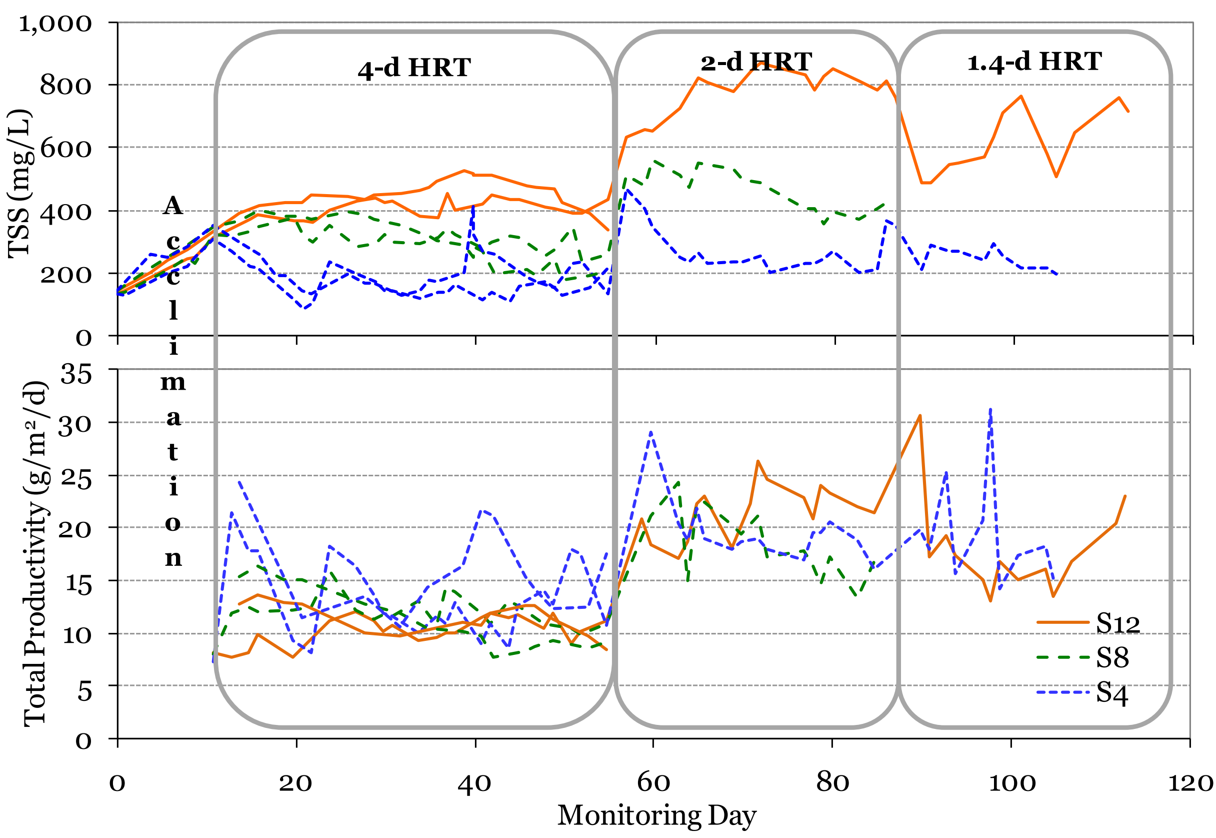

Figure 5-5. Mixture TSS and Total Productivity of Microbial SBRs Operated at Different SRTs and HRTs. (Note: 4-d HRT period shown in duplicate.)

S12 and S4 were further monitored at 1.4-d HRT and maximum productivities of 31 g/m²/d were observed (Table 5-4). However, mixture TSS was variable and resulted in somewhat lower mean total productivities of 18.0 and 19.5 g/m²/d for S12 and S4, respectively. This shortcoming was likely due to a combination of DO limitation (Section 5.3.3), 27% lower average $sCOD_i$ during this period (resulting in only a marginal increase in loading from 30.7 to 32.0 g/m²/d $sCOD_i$ [Table 5-3]), and unintended solids discharge from S12 (Section 5.4).

In addition to high productivity, high mixture TSS is preferred in biomass production systems because less dewatering is required during harvesting to make it more cost-effective. Mixture TSS was impacted by HRT since it affected nutrient availability and by SRT since it determined microbial growth rate (Figure 5-5). TSS was greatest at longest SRT since more biomass, although growing slower, was retained longer in the SBRs. A maximum TSS of 871 mg/L was achieved for S12 during 2-d HRT indicating lowest anticipated dewatering requirements using these operational conditions.

5.2.2 pH and DO Concentrations

Photosynthesis, respiration, and absorption of atmospheric oxygen impacted pH and DO levels of the cultures. Microalgae photosynthesised during the light period which increased pH and DO while ongoing respiration during the dark period reduced these levels causing the diurnal

fluctuations shown in Figure 5-6. Mean pH for each SBR was nearly neutral (i.e., 7.1-7.6 SU) and similar to that of feed water (i.e., 7.4 ± 0.2 SU [Table 2-1]). Similar pH trends and magnitudes were observed across cultures (Table 5-5) indicating that comparable levels of dissolved CO_2 existed for photosynthesis due to pH's influence on carbonate chemistry (Liehr et al. 1988).

Shorter HRT generally resulted in lower DO due to increased oxygen demand for wastewater degradation. Longer SRT enabled greater mixture TSS, which reduced light penetration and photosynthetic oxygenation (i.e., DO of S12 < S8 < S4 at 4-d HRT [Figure 5-6 and Table 5-5]) while increasing endogenous respiration. Endogenous respiration occurred when nutrients such as C were depleted from the growth medium, and microbes oxidised their own resources (i.e., requiring O_2) for survival (Gray 2004). Consequently, DO was lower than observed previously at 8-d HRT (Section 4.2.2), and bacterial metabolism was probably limited at < 4-d HRT due to minimal (< 0.5 mg/L) DO availability (Gray 2004). However, < 21% DO saturation (i.e., < 1.9 mg/L at 19 °C) was recommended for microalgae, and even a 14% increase in photosynthetic efficiency was demonstrated near anaerobiosis by Becker (1994) possibly due to faster CO_2 fixation and/or less photooxidative damage occurring at lower DO (Suh and Lee 2003). Therefore, the lower DO levels of these operating conditions (Table 5-5) appeared more favourable for microalgal growth compared to those at longer retention times (Section 4.2.2).

Although DO measurements indicated that cultures were anaerobic at times (Figure 5-6 and Table 5-5), continual atmospheric oxygen absorption and daytime photosynthetic oxygenation occurred. Maximum atmospheric oxygen absorption rates of 4 to 5.7 mg/L/h have been reported for HRAP-type systems with 28- to 35-cm depth at 24 °C (Grobbelaar et al. 1988); (El Ouarghi et al. 2000). Since culture DO was usually quite low (i.e., $M \leq 1$ mg/L for all conditions except M=1.9 mg/L for S4 at 4-d HRT [Table 5-5]), atmospheric oxygen absorption was nearly constant across cultures while being slightly less for S4 at 4-d HRT due to a smaller oxygen deficit. Assuming a constant value of 4 mg/L/h (i.e., from (Grobbelaar et al. 1988)] for 28-cm depth, 40-rpm mixing with rotating, submerged, stainless steel blade, and 24 °C vs. 32.5-cm depth, 50-rpm mixing with rotating, submerged, stainless steel blade, and 19 °C for this experiment), maximum oxygen transfer into these cultures would amount to 31 g/m^2/d. Moreover, photosynthetic oxygenation of 1.92 g DO/g dw (i.e., as measured by Grobbelaar et al. [1988]), would have produced 10-23 g/m^2/d (i.e., 2.1-4.9 mg/L/h during light periods). (Mean total productivities [Table 5-4] and corresponding mean microalgal biomass contents [Section 6.2.2] were used to calculate this range.) Together, an estimated oxygen input of 41-54 g/m^2/d supported microbial respiration and primary wastewater degradation to varying degrees without supplemental aeration as evident by modelling results (Section 5.3), COD removal (Section 5.6.1), and species cohabitation (Section 6.1).

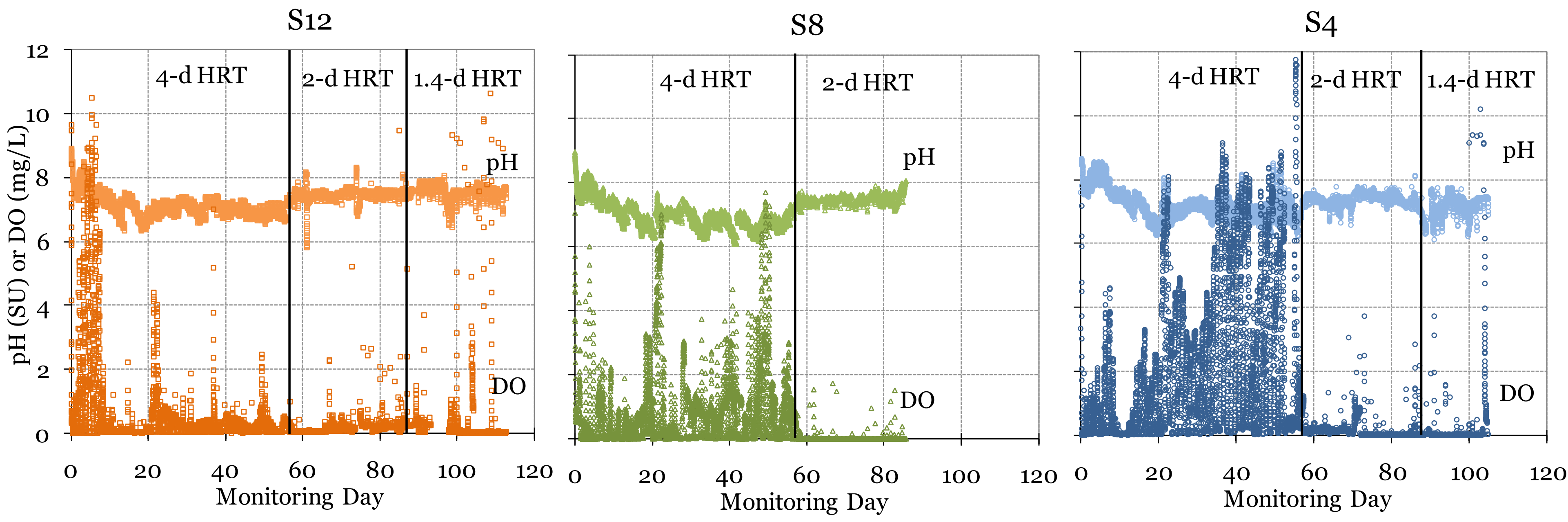

Figure 5-6. Continuous pH and DO Measurements of Microbial SBRs Operated at Different HRTs and SRTs.

Table 5-5. Summary of pH and DO Measurements of Microbial SBRs Recorded at 15-min Intervals.

Parameter	SBR	Minimum	Maximum	4-d HRT			2-d HRT			1.4-d HRT		
				M	±	*SD*	*M*	±	*SD*	*M*	±	*SD*
pH (SU)	S12	5.6	9.1	7.2	±	0.4	7.5	±	0.2	7.6	±	0.2
	S8	6.1	9.1	7.1	±	0.4	7.5	±	0.1		—	
	S4	6.1	10.5	7.3	±	0.5	7.4	±	0.2	7.2	±	0.3
DO (mg/L)	S12	0.0	13.7	0.4	±	1.2	0.8	±	2.4	0.2	±	1.0
	S8	0.0	13.0	0.6	±	1.1	0.0	±	0.1		—	
	S4	0.0	12.7	1.9	±	2.3	0.1	±	0.3	0.2	±	0.8

5.3 Assessment of Limitations

5.3.1 Generalisations and Simplifications

The complexity of the microbial communities in the SBRs (i.e., numerous species interacting at various trophic levels) and the multiple metabolic strategies being employed (i.e., potentially varying by species, nutrients, and light/dark periods) likely limited microbial growth in different ways at different times for different species. Therefore, several non-trivial simplifications and assumptions were made (Table 5-6) to facilitate modelling efforts necessary to explore possible productivity limitations for the predominant microbes—photoautotrophic microalgae and heterotrophic bacteria.

Table 5-6. Simplifications and Assumptions Used to Explore Biomass Productivity Limitations.

Clause	Description	Validity
S1	TSS consisted of 90% organic matter, all of which was viable microalgae and bacteria.	In actuality, cell viability decreases as SRT increases because more energy is used for cell maintenance (Orhon et al. 2009).
S2	All microalgae were photoautotrophs and all bacteria were heterotrophs.	Other metabolic pathways were likely including microalgal heterotrophy (Becker 1994).
A1	Influent TSS was completely degraded and assimilated into biomass and/or enmeshed within flocs.	Assumed for this wastewater treatment model as per Eisenberg (1981) and also based on 91% biodegradability of tCOD (Orhon et al 2009).
A2	Anaerobic heterotrophy did not occur.	No malodours were observed (e.g., H_2S).
A3	Nitrification and denitrification were negligible.	Oxidation of all COD would have occurred before these processes, and DO levels were generally low.
A4	Chl *a* constituted 1.5% of microalgal biomass.	Chl *a* content of microalgae can vary from 1-2% and can be biased by culture conditions (APHA 2005).

Note: S = simplification; A = assumption.

5.3.2 Nutritional Assessment

For an initial assessment, primary wastewater quality was examined to determine if there were limiting concentrations of any major nutrients possibly affecting microbial growth. Microalgae and bacteria have different chemical formulas depending on species and growth conditions, but, overall, they have similar compositions of major nutrients: 50-59% C, 26-37% O, 6-12% N, 5-8% H, and < 1% P as summarised by Humenik and Hanna (1971). Requirements of O and H are often met through bacterial degradation of organic compounds, while other minor elements (e.g., K, Na, Ca) and trace elements (e.g., Co, Mn, Zn) often exist in sufficient quantity in municipal wastewater (Gerardi 2006). Thus, C, N, and P contents could potentially limit productivity.

Influent primary wastewater COD ($tCOD_i$), which was indicative of C content ($r[36]=0.9244$), was evaluated to determine unrestricted microbial growth requirements for N and P. Initial total

biodegradable COD ($bCOD_o$) at the start of the RP was calculated according to Equation 5-1. Assuming 91% of $tCOD_i$ is biodegradable and/or hydrolysable (Orhon et al. 2009) and, therefore, presumably tied up in biomass, effluent soluble COD ($sCOD_t$) was used to approximate the COD remaining for microbial growth prior to effluent discharge. Biomass yields were then calculated for each operational period using Equation 5-2. Organic contents were similar across HRTs and SRTs indicating constant production of inert solids (Section 6.2.1).

$$bCOD_o = f_s \times \frac{tCOD_i}{HRT} + (1 - 1/HRT) \times (sCOD_t) \tag{5-1}$$

$$Y = \frac{(X_t - X_o) \times r}{bCOD_o - sCOD_t} \tag{5-2}$$

Where: *$bCOD_o$ = initial total biodegradable COD concentration at time 0 (mg/L)*
f_s=biodegradable fraction of tCOD (0.91; Orhon et al 2009)
$tCOD_i$ = influent tCOD concentration (mg/L) (Section 5.1.2.2)
HRT = hydraulic retention time (d)
$sCOD_t$ = effluent sCOD concentration at time t (mg/L) (Section 5.6.1)
Y = biomass yield (g cell COD/g COD_{used})
X = mixture TSS concentration (X_o at time 0; X_t at time t) (mg/L)
r = ratio of 1.58 g COD/g TSS for biomass (Appendix C)

Yields of 0.76-0.95 g cell COD/g COD_{used} during 4-d HRT (Figure 5-7) were almost 50% greater than those of conventional AS systems (i.e., 0.64 g cell COD/g COD_{used} [Orhon et al. 2009]). COD directly supported bacteria, which indirectly supported microalgae through fixation of respired CO_2. Hence, reduced loss of feed water C and any atmospheric CO_2 fixation contributed to the enhanced yields. Lower yields of 0.49-0.63 g cell COD/g COD_{used} at shorter HRTs (Figure 5-7) indicated less efficient use of feed water C probably due to greater (DO) growth limitation.

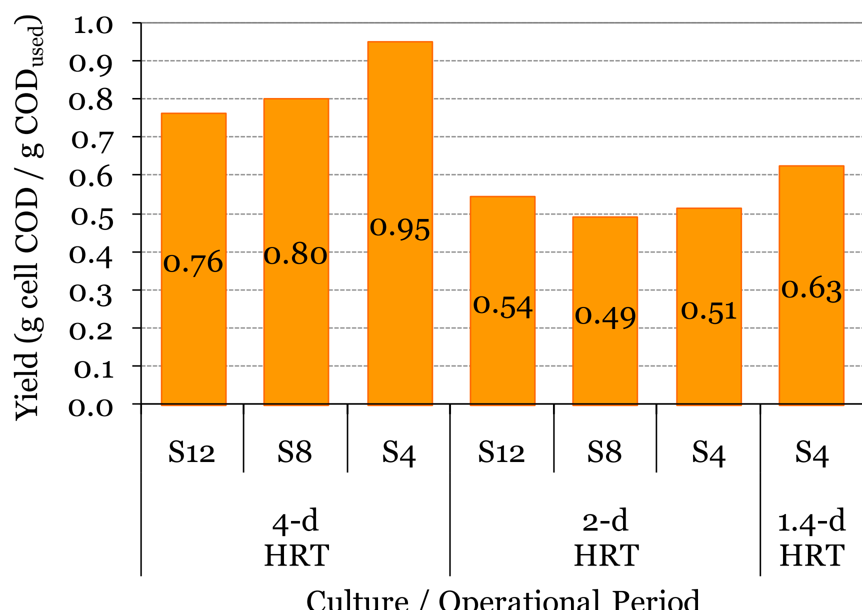

Figure 5-7. Yields of Microbial SBRs Operated at Different HRTs and SRTs.

Based on a maximum yield of 0.95 g cell COD/g COD_{used} obtained for S4 during 4-d HRT, maximum N and P requirements for microbial growth were 25 ± 8 mg/L and 6 ± 2 mg/L, respectively (Table 5-7). The requirements were even less for the majority of the cultures due to lower biomass yields. Comparing the values with those of primary wastewater (i.e., 44 mg N/L and 6.5 mg P/L [Table 2-1]) indicated that sufficient N and P probably existed for growth, and C was identified as the most plausible nutrient limitation. Otherwise, the next most likely candidate was P, but possibly only for S4 during 4-d HRT.

Table 5-7. Microbial N and P Requirements for Growth Based on tCOD of Primary Wastewater.

Parameter	Unit	Value	Reference
Influent Total COD ($tCOD_i$)	mg/L	360 ± 119	Table 5-3
Biodegradable Fraction of tCOD (f_s)	g biodegradable tCOD/g $tCOD_i$	0.91	Orhon et al. 2009
Maximum Biomass Yield (Y)	g cell COD/g COD_{used}	0.95	Figure 5-7
Organic N Fraction of Biomass (i_{XN})	g N/g cell COD	0.08	Orhon et al. 2009
Calculated N Required	mg/L	25 ± 8	$=tCOD_i*f_s*Y*i_{XN}$
Organic P Fraction of Biomass (i_{XP})	g P/g cell COD	0.02	Orhon et al. 2009
Calculated P Required	mg/L	6 ± 2	$=tCOD_i*f_s*Y*i_{XP}$

5.3.3 Metabolic Assessment of DO and Light

Environmental conditions were subsequently examined to determine productivity limitations. The light and dark periods (i.e., also referred to as day and night, respectively) affected different metabolic conditions for the microbes, primarily on account of the oxygenation capacity of the microalgae (Section 5.2.2). DO limitation was assessed in-depth by examining 5-d data subsets for each culture period when stable (TSS) conditions existed (i.e., monitoring days 30-35, 65-70, and 98-103 for 4-, 2-, and 1.4-d HRTs, respectively). Hence, culture S12 during 1.4-d HRT was omitted from this evaluation due to solids washout. The accuracy of the DO meters was ± 0.2 mg/L, so values < 0.2 mg/L were treated as 0 mg/L for the purposes of this analysis. Accordingly, all cultures were predominantly anaerobic at night (Figure 5-8). Photoautotrophic light limitation caused heterotrophic DO limitation at night despite atmospheric oxygen absorption since AS treatment can be impaired at < 0.5 mg/L DO (Gray 2004). Meanwhile, absence of odours (e.g., H_2S) discounted anaerobic heterotrophy.

Heterotrophic growth and respiration countered daytime photosynthetic oxygenation. During heterotrophy, substrate (COD) was first converted into energy and biomass during growth, and then the biomass was oxidised during endogenous respiration (i.e., both processes preferentially requiring O_2 as an electron acceptor) to generate energy for cell maintenance (Orhon et al. 2009). Since some COD remained unoxidised and culture DO was generally low, nitrification and denitrification were assumed negligible. Peak and subsequent decrease of DO *prior* to the dark period during some 4-d HRT cycles (e.g., at arrows on days 30-34; Figure 5-8) may have

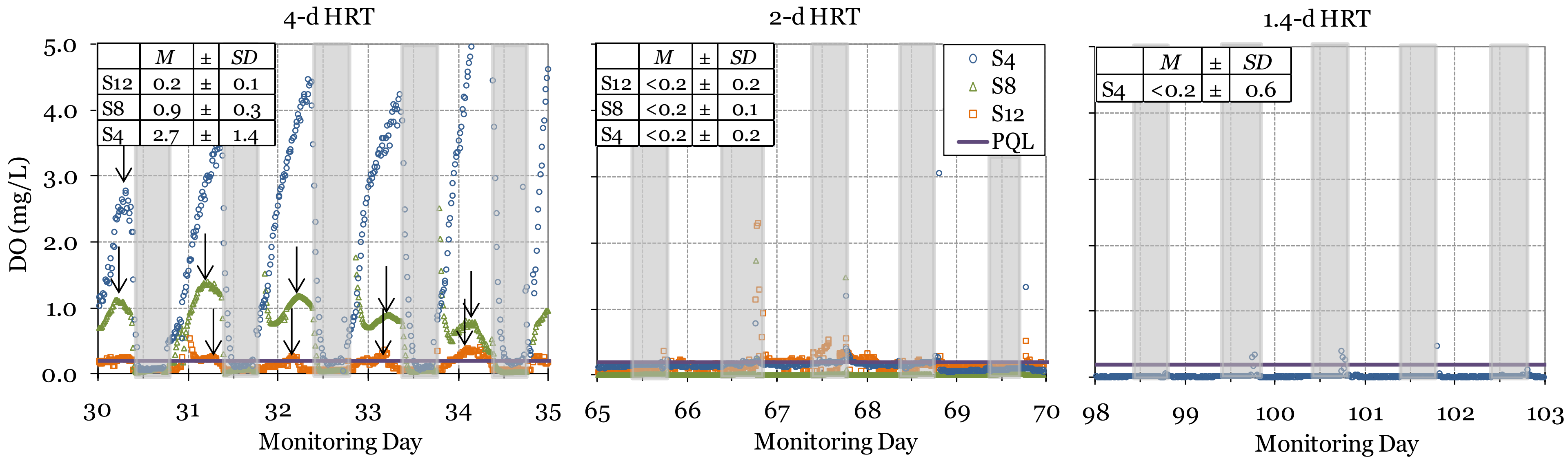

Figure 5-8. Subsets of Low-Range DO Measurements of Microbial SBRs over Stable, 5-d Periods for Each HRT to Determine Heterotrophic Limitation. (Note: shaded areas indicate dark period; 0.2-mg/L DO meter practical quantitation limit [PQL] discounted lower values; daytime DO tabulated in mg/L [*M*±*SD*]; heterotrophic oxygen demand exceeded microalgal oxygen production at point indicated by ↓; S12 omitted from 1.4-d HRT period due to instability.)

indicated increased bacterial demand possibly due to a longer lag phase than microalgae following primary wastewater addition at the start of the light period and/or reduced microalgal photosynthesis due to light limitation from shading by increasing TSS.

Cultures S4 and S8 were clearly aerobic (i.e., > 0.5 mg/L DO) and S12 was micro-aerobic (i.e., < 0.5 mg/L DO) during 4-d HRT (Figure 5-8). However, during daytime of 2- and 1.4-d HRTs, increased oxygen demand for carbonaceous oxidation rivalled DO production by microalgae, effectively causing all cultures to be limited at < 0.2 mg/L DO. These conditions showed that requirements for bacterial growth and respiration exceeded microalgal oxygenation at short (< 4-d) HRT. However, DO measurements reflected mixture oxygen levels only—not what was actually produced by microalgae and directly available to bacteria in flocs. In reality, efficient oxygen transfer was occurring between microalgae and bacteria because of their close proximity within the flocs and symbiosis as modelled by Humenik and Hanna (1970). This relationship was further supported by sCOD removal (Section 5.6.1). So, although 2- and 1.4-d HRT cultures appeared (nearly) anaerobic, it was because photosynthetic (and atmospheric) oxygen was immediately scavenged by bacteria within the flocs for their own metabolism under DO-limiting conditions before mixture DO measurements were impacted.

5.3.4 Models and Implications

Culture conditions likely affected microbial growth via the Monod Equation (5-3) as modelled by others (Goldman and Carpenter 1974; Brune and Novak 1981; Liu et al. 2005). Ratios of μ/μ_m indicated proportions of actual growth rate to maximum growth rate attainable. These ratios were valuable to compare different scenarios to determine biomass growth limitations—cultures growing at lower μ/μ_m (or % of μ_m) were more growth limited than cultures growing at higher μ/μ_m (or % of μ_m). Two applications of the Monod Equation were considered—C-limited growth (Model 1) and DO-limited growth (Model 2) over the duration of the growth cycle while ignoring potential concentration gradients within the flocs. Since fed-batch reactors were used for these experiments, nutrients such as COD supplied at the start of the light period became depleted, resulting in decreasing bacterial growth over the day depending on availability of the limiting factor. Therefore, ranges of μ/μ_m were calculated from influent and effluent COD (i.e., $bCOD_o$ [Equation 5-1] and $sCOD_t$ [Section 5.6.1], respectively) using Equation 5-3. Growth ranged from 64-93% of μ_m (Model 1; Table 5-8) based on a half-saturation constant (K_s) of 20 mg COD/L for heterotrophy (Henze 2000).

Daily DO means, rather than ranges, were used to calculate DO-limited growth ratios because they were more informative than minimums (i.e., always 0 mg/L) and maximums (i.e., generally varied daily) (Figure 5-8). Thus, DO resulted in mean growth ratios of 50-93% of μ_m during 4-d HRT, but severely limited growth at < 50% of μ_m during 2- and 1.4-d HRTs (Model 2; Table 5-8) using a K_s value of 0.2 mg O_2/L for heterotrophy (Henze 2000).

$$\mu = \frac{\mu_m C}{K_s + C} \qquad 5\text{-}3$$

Where: μ = *specific growth rate* (d^{-1})
μ_m = *maximum specific growth rate* (d^{-1})
C = substrate concentration (mg/L)
K_s = *half-saturation constant for C, when* $\mu = \mu_m/2$ *(mg/L)*

Table 5-8. Daytime Microbial Growth Rate Ratios Modelled for C and DO Limitation.

Monod Model Description	SBR	μ/μ_m (%) @ 4-d HRT	μ/μ_m (%) @ 2-d HRT	μ/μ_m (%) @ 1.4-d HRT
Model 1: C-limited growth for K_s=20 mg/L COD	S12	85-64*	93-73	NA
	S8	85-64*	93-77	NA
	S4	86-70*	93-78	91-73
Model 2: DO-limited growth for K_s=0.20 mg/L DO	S12	50	< 50*	NA
	S8	82	< 50*	NA
	S4	93	< 50*	< 50*

*Condition likely predominating; NA = not available since stable condition not achieved.

Based on the above approaches for modelling C- and DO-limited growth, comparison of μ/μ_m ratios determined the prevailing limitation. C levels affecting growth at 64-86% of μ_m compared with 82-93% of μ_m for DO indicated that C limitation dominated in S4 and S8 during 4-d HRT (). Determining heterotrophic growth limitation for S12 during 4-d HRT was not as obvious. Growth rates at 64-85% of μ_m for C and < 50% of μ_m for DO were calculated (Table 5-8). While this would ordinarily indicate DO limitation, accuracy of the DO meters at very low levels (i.e., < 0.2 mg/L) was not reliable. Moreover, productivity actually increased at greater C loading (for all cultures) from 4- to 2-d HRT (Table 5-4) while DO simultaneously decreased (Table 5-5) to indicate that C was the overriding limitation. During 2- and 1.4-d HRTs, C affecting growth at 73-93% of μ_m compared with < 50% of μ_m for DO in all SBRs (Table 5-8) indicated that DO limitation dominated growth of these cultures.

Daytime microalgal growth limitation was not modelled due to limited data (e.g., absence of dissolved CO_2 measurements). However, examination of conditions impacting light and CO_2 availability could reveal the limiting factor. Mean mixture TSS increased for each daily cycle by about 8, 13, and 29% for S12, S8, and S4 cultures, respectively, regardless of HRT period (i.e., due to constant mean growth rate) (Table 5-4). Although light supply was constant from the bulbs during the day, light penetration inherently decreased as TSS increased. Meanwhile, CO_2 production presumably increased from bacterial growth and endogenous respiration. These processes inferred that microalgal growth was more likely to become light limited over the course of the day, which would have depended on species' specific K_s values (Richardson et al. 1983). Regan and McKinney (1977) also found that microalgae cultured outdoors on domestic wastewater were light limited.

Although purely photoautotrophic metabolism was assumed for microalgal growth, mixotrophic metabolism, where microalgae can switch from photoautotrophy to heterotrophy depending on availability of light, CO_2, and organic C, was probably more realistic as observed by others (Richmond 2004; Perez-Garcia et al. 2010). For instance, *Scenedesmus* spp. and *Chlorella* spp. can switch from autotrophy when growing on CO_2 in light to heterotrophy using organic C in darkness (Becker 1994). The total estimated oxygen input of 41-54 g/m^2/d (Section 5.2.2) may further support this possibility since complete degradation of all incoming BOD would have required only 12-36 g/m^2/d (i.e., assuming 0.75 g DO/g BOD for synthesis and 0.048 g DO/g TSS for endogenous respiration [Gray 2004]). In reality, minimal excess DO existed and not all BOD was degraded, which indicates that oxygen production was overestimated potentially owing to some microalgal heterotrophy occurring under light limiting conditions and/or a smaller photoautotrophic contribution resulting from uncertainty in Chl *a* data (Section 6.2.2). However, this metabolic flexibility would not have substantially impacted the results of this assessment since stable conditions were reached in which microalgae and bacteria assumedly had equivalent growth rates.

Productivity of cultures was sensitive to growth limitations. Nutritional and metabolic assessments indicated greatest potential for C and DO limitation to heterotrophic bacteria. Modelling these factors using the Monod Equation (5-3) suggested that 4-d HRT cultures were C limited and 2- and 1.4-d HRT cultures were DO limited. Meanwhile, since light penetration decreased as TSS and CO_2 production increased during the day, photoautotrophic microalgae were presumably light limited for all conditions. Assessments only required K_s values and substrate concentrations and were not impacted by microbial fractions, so outcomes were fairly sound depending on the applicability of K_s values, which have been widely used in wastewater treatment modelling (Henze 2000).

5.4 Biomass Settleability

5.4.1 Performance in Microbial SBRs

Natural sedimentation of suspended solids occurred daily in the SBRs during the darkened 60-min settling periods due to the development of stable, compact flocs and bioflocculation (Section 4.3). Settleability ranged from 36-98% and was generally greater and more consistent at longer SRT and shorter HRT (Figure 5-9). Only six (out of 185) measurements yielded < 60% settleability, and the exceptions were all for S4 while operated at 4-d HRT (i.e., without biomass recycling). Reduced settleability during days 40-55 resulted most markedly in S4 presumably because it was not as resilient to changes in influent COD as the other SBRs due to its lower TSS.

There was an overall increase in settleability concurrent to a decrease in growth rate at increasing SRT/HRT ratio (Figure 5-10), which demonstrated the value of biomass recycling.

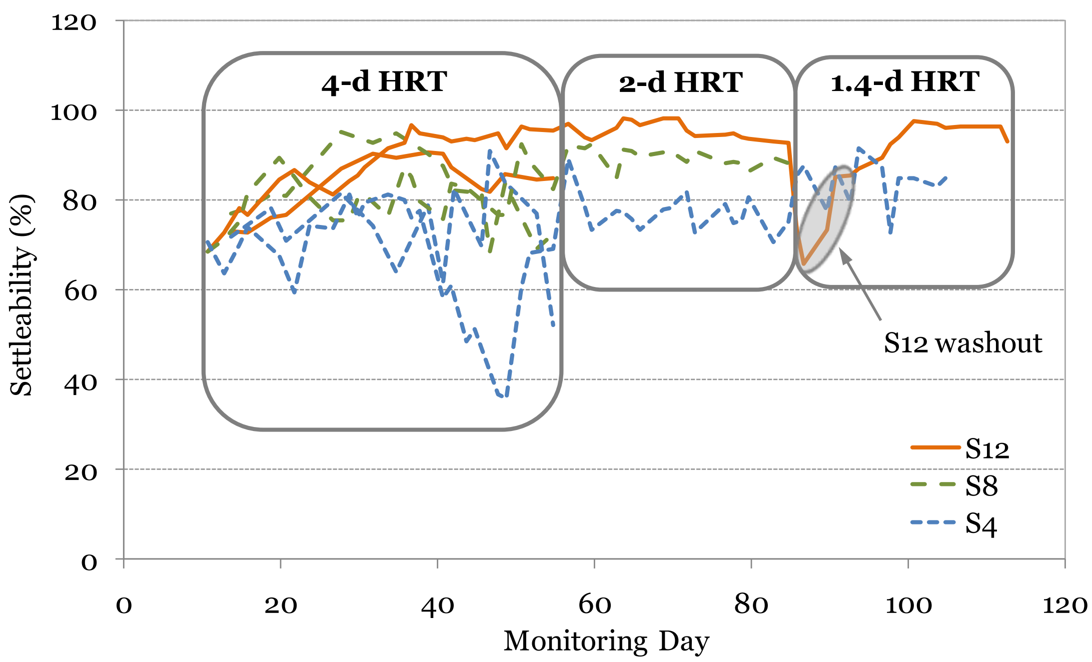

Figure 5-9. Settleability of Microbial SBRs Operated at Different SRTs and HRTs.

(Note: 4-d HRT period shown in duplicate.)

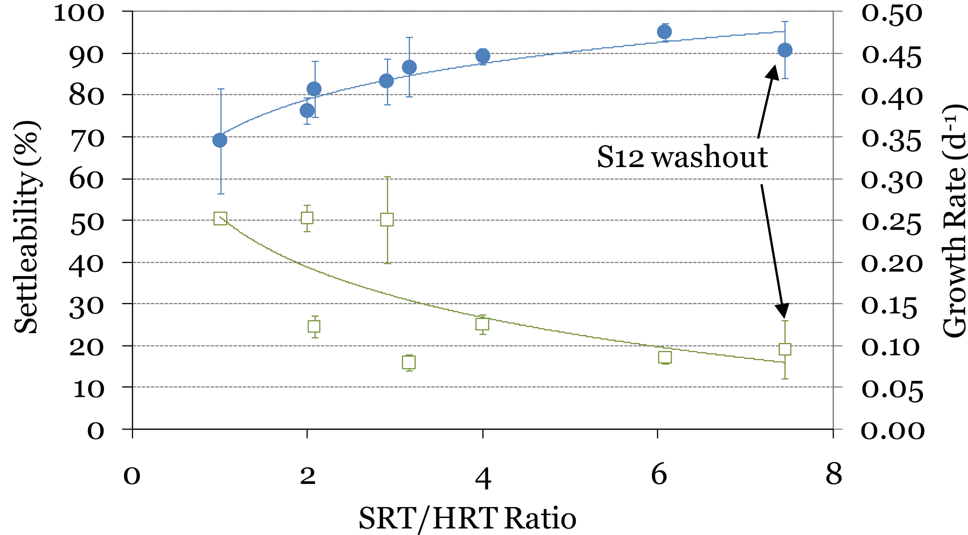

Figure 5-10. Effect of SRT/HRT Ratio on Mean Settleability (•) and Microbial Growth Rate (□).

These trends probably occurred for two reasons: 1) longer SRT enabled greater microbial agglomeration due to slower growth (i.e., 0.08 d^{-1} at 12-d SRT vs. 0.25 d^{-1} at 4-d SRT), and 2) greater selective pressure existed for settleable microbes since unsettleable supernatant microbes were wasted. Settleable biomass recycling favoured the growth of aggregating microbes (e.g., *Micractinium* spp.) similarly to the AS process, since the competitive advantage of fast growing, unicellular microbes (e.g., *Chlorella* spp.) was reduced by selectively wasting them during supernatant discharge as demonstrated by Weissman and Benemann (1979). This strategy suggested that shorter HRT (and longer SRT) exuded greater selection pressure for

settleable microbes to dominate since more supernatant and unsettleable microbes were wasted, which was consistent with increased settleability in all SBRs from 4- to 2-d HRT. When further reducing HRT to 1.4 d, an operational issue surfaced in S12—the volume of settled solids was now higher than the new supernatant discharge level, which caused them to be discharged along with supernatant resulting in lower apparent settleability (Figure 5-9). This excessive solids wasting in S12 also meant that 12-d SRT was not able to be maintained at 1.4-d HRT (i.e., *M*=10.3, *SD*=2.8 d [Table 5-2]). SRT control was not an issue for S4 since it contained less solids than S12 (i.e., about 200 vs. 800 mg/L before the operational change to 1.4-d HRT), so they did not interfere with the supernatant discharge port. Hence, further increased settle-ability in S4 from 76% at 2-d HRT to 83% at 1.4-d HRT was consistent with greater selection pressure, but it was not apparent in S12 due to the unintended discharge of settled solids.

The SBRs demonstrated very good settleability with biomass recycling—values ranged from 68-98% (with < 10% *SD*) (Table 5-4) which may render sedimentation more efficient and consistent than reported by previous studies. For example, under outdoor summer conditions without biomass recycling, Eisenberg et al. (1981) obtained 75-99% settleability only after 24 h, and Park and Craggs (2010) obtained 63 ± 14% after 3 h. Results from these retention time experiments demonstrated that gravity sedimentation can be an effective primary harvesting method for microbial biomass production systems. Further, settleable biomass recycling increased efficiency and reliability of sedimentation as SRT increased and HRT decreased (i.e., SRT/HRT ratio increased).

5.4.2 Complementary Investigations

Sedimentation time and container were examined for their influence on settleability. Visual observations from previous experiments showed that solids settled quickly within the 60-min sedimentation period (Section 4.3). Hence, the effect of sedimentation time was quantified by collecting supernatant TSS samples after 15, 30, and 45 min in Imhoff Cones and comparing them to mixture TSS and 60-min supernatant TSS values to determine sedimentation efficiency (Figure 5-11a). Results agreed with previous observations and indicated that sedimentation time of future studies could be reduced from 60 to 30 min with comparable settleability. This change was not adopted in this study in order to maintain consistency with previous experiments.

Sedimentation container (i.e., size, material, and/or shape) also appeared to have an effect on settleability. Comparison of supernatant TSS from 1-L, plastic Imhoff Cones and 28-L, steel SBRs demonstrated poorer settleability in Imhoff Cones (*M*=79%, *SD*=6%) than SBRs (*M*=82%, *SD*=7%) with only one exception (as seen above the equality line) (Figure 5-11b). Thus, actual settleability of the cultures may have been underestimated (i.e., 3% difference) previously (Sections 3.2 and 4.3), but it was accurate for this study. In contrast, Eisenberg et al. (1981) found greater settleability in Imhoff Cones after 24 h (*M*=78%) compared to settling ponds after

48 h (M=71%). Therefore, it is worthwhile to verify comparability of actual and estimated settleabilities in order to give a reliable indication of actual harvestable yield.

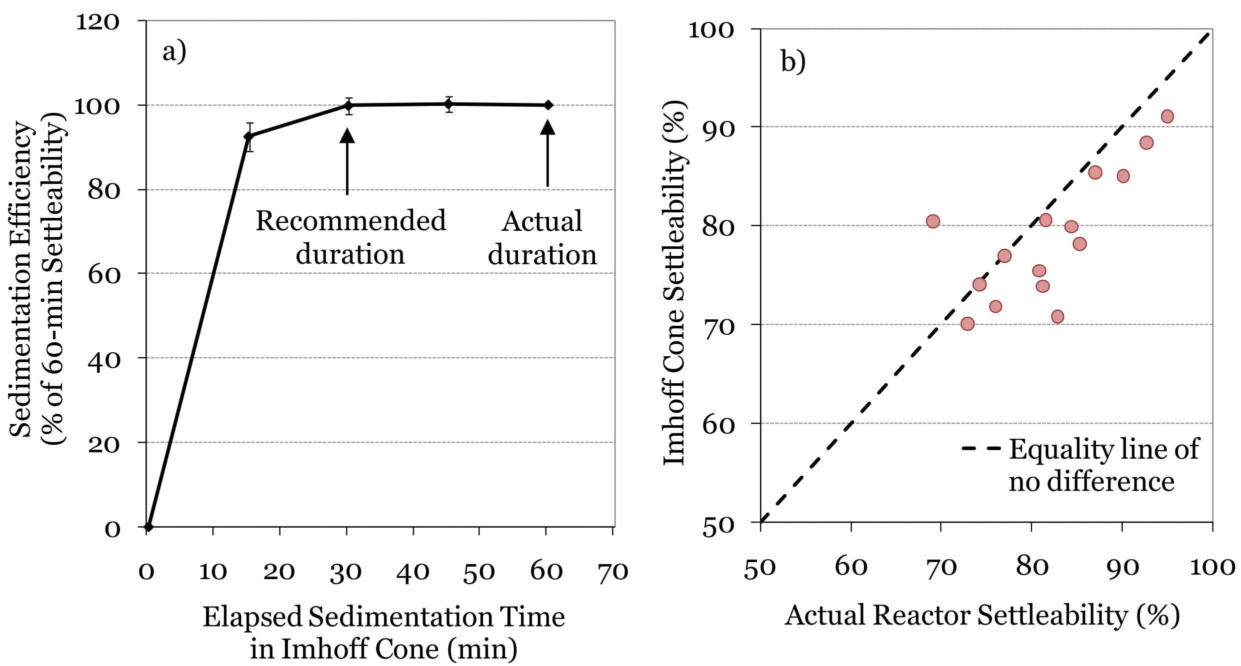

Figure 5-11. Effects of a) Sedimentation Time (n=3) and b) Settling Container on Settleability (n=15).

5.5 Settleable Productivity

Settleable productivity was simply total TSS productivity multiplied by settleability—essentially a combination of two critical performance data used to estimate the amount of solids harvestable by sedimentation. During 4-d HRT, total productivities increased (i.e., from 10.7-14.2 g/m²/d), but settleabilities decreased (i.e., from 87-69%) with decreasing SRT from S12-S4, so similar settleable productivities were obtained at 9.3-9.9 g/m²/d (Figure 5-12). Shortening to 2-d HRT increased total productivities for all SBRs (i.e., 18.5-21.6 g/m²/d) while 76-95% settleabilities affected corresponding settleable productivities of 20.6, 16.5, 14.9 g/m²/d for S12, S8, and S4, respectively (Figure 5-12). Therefore, both variables were equally important at 4-d HRT, but settleability was the determining variable at 2-d HRT for greater settleable productivity since total productivities were similar. These values agreed with the range of 15.8-30.7 g VSS/m²/d settleable productivity in outdoor HRAPs at 2-d HRT calculated from Eisenberg (1981). Total productivities and settleabilities at 1.4-d HRT were affected operationally (Sections 5.2.1 and 5.4), which reduced settleable productivity to 16.2 g/m²/d for S12, but increased it to 16.1 g/m²/d for S4 (Figure 5-12). Park and Craggs (2010) achieved mean settleable productivity of 14.7 g VSS/m²/d from a HRAP operated at 4-d HRT with CO_2 addition in NZ. Although mean settleable TSS productivities of 9.3-9.9 g/m²/d from these experiments at 4-d HRT were expectedly lower than this value without CO_2 addition, it appears that greater wastewater loading displaced the need for CO_2 addition to maximise productivity. Mean

settleable TSS productivity was optimised at 20.6 g/m²/d using 12-d SRT and 2-d HRT of primary wastewater despite reduced light penetration owing to excellent settleability and efficient use of nutrients to the point of DO limitation. This value is the highest that has been reported for NZ conditions, but the consistent laboratory environment, free from climatic variations and other outdoor influences, potentially overestimated realistic yield of large-scale systems.

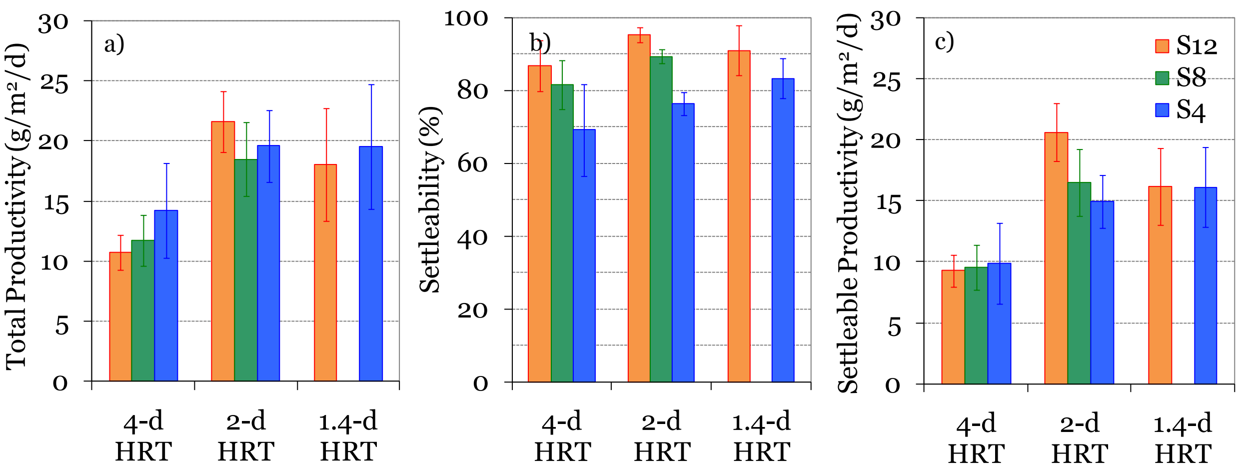

Figure 5-12. Critical Parameter Means for a) Total Productivity, b) Settleability, and c) Settleable Productivity of Microbial SBRs Operated at Different SRTs and HRTs.

5.6 *Wastewater Treatment*

Demonstrating wastewater treatment capacity for these cultures was not a priority since it was already investigated previously. Nonetheless, since sCOD and supernatant TSS were measured to assess other essential parameters (i.e., C loading and settleability), they were also examined for an indication of wastewater treatment.

5.6.1 *Soluble COD Removal*

Mean sCOD removal efficiency (*R*) (Equation 5-4) decreased from 71-77% at 4-d HRT to 62% at 1.4-d HRT (Table 5-9). Overall, $sCOD_t$ ranged from 16-114 mg/L and was normally lower at higher SRT (Figure 5-13). Similarly, Cromar and Fallowfield (1997) achieved COD removal of 55-60% in outdoor HRAPs. $sCOD_t$ values were lower than those (i.e., *M*=152 mg/L) obtained by

$$R = 100 \times \frac{sCOD_i - sCOD_t}{sCOD_i} \qquad 5\text{-}4$$

Where: *R = removal efficiency (%)*

$sCOD_i$ = influent sCOD concentration (mg/L) (Table 5-3)

$sCOD_t$ = effluent sCOD concentration (mg/L) (Table 5-9)

Table 5-9. Wastewater Treatment by Microbial SBRs Operated at Different SRTs and HRTs.

Parameter	SBR	*n*	Minimum	Maximum	*M* ± *SD*	*R*
4-d HRT						
$sCOD_t$ (mg/L)	S12	21	16	53	36 ± 10	77
	S8	21	22	53	36 ± 9	77
	S4	21	22	69	46 ± 12	71
Supernatant TSS (mg/L)	S12	38	14	113	55 ± 28	-
	S8	38	17	92	54 ± 20	-
	S4	37	16	109	54 ± 27	-
2-d HRT						
$sCOD_t$ (mg/L)	S12	7	34	80	55 ± 18	72
	S8	8	46	86	66 ± 13	66
	S4	7	47	114	72 ± 22	63
Supernatant TSS (mg/L)	S12	16	15	58	38 ± 16	-
	S8	17	39	78	49 ± 9	-
	S4	16	46	94	60 ± 13	-
1.4-d HRT						
$sCOD_t$ (mg/L)	S12*	6	30	76	54 ± 18	62
	S4	4	41	62	54 ± 9	62
Supernatant TSS (mg/L)	S12*	13	19	130*	52 ± 32	-
	S4	10	23	81	41 ± 17	-

*SBR was not able to be held at constant SRT during this period due to solids washout.

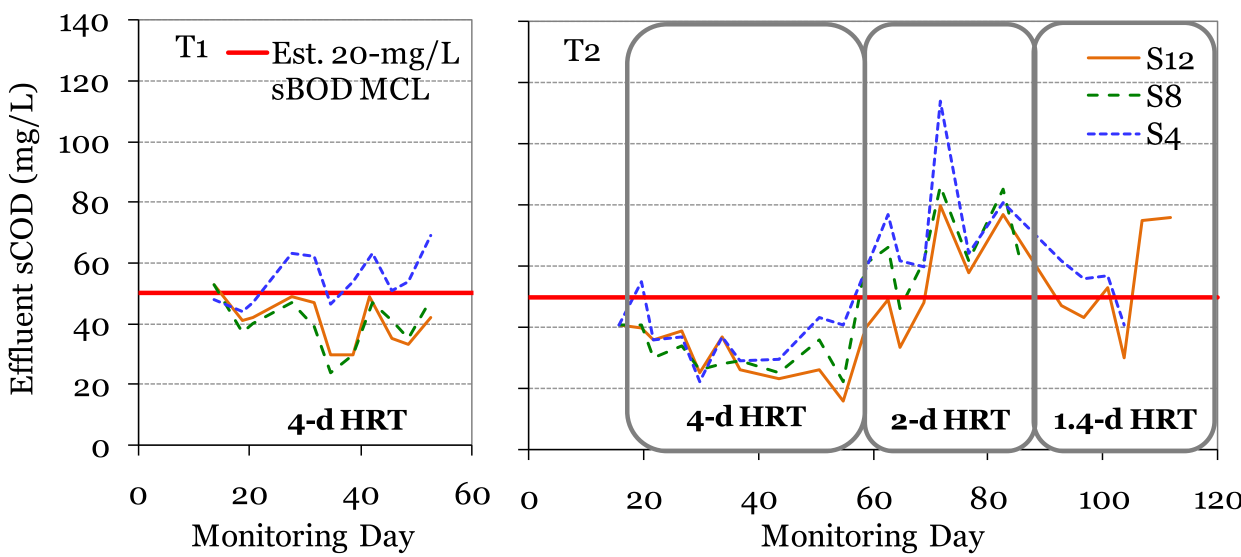

Figure 5-13. Effluent sCOD of Microbial SBRs Operated at Different SRTs and HRTs. (Note: MCL for discharge compliance estimated based on sCOD/sBOD ratio of 2.5 in primary wastewater.)

Travieso et al. (2006) using microbial biomass to treat 451 mg/L tCOD wastewater with a 4-d HRT. The MCL of soluble biochemical oxygen demand (sBOD) for wastewater discharge (per CRC051724) is 20 mg/L for compliance. Accordingly, based on a sCOD/sBOD ratio of 2.5 in primary wastewater, values often exceeded the estimated equivalent sCOD MCL of 50 mg/L

(Figure 5-13). $sCOD_t$ was greatest during 2-d HRT probably due to DO limitation as well as greater $sCOD_i$ during this period. To best meet the sBOD MCL, 4-d HRT with biomass recycling is recommended, but this may result in lower total productivity.

5.6.2 Supernatant TSS

Laboratory measurements of primary wastewater TSS ranged from 65-100 mg/L indicating potential microbial solubilisation of organic solids during storage (e.g., similarly to $sCOD_i$ [Appendix B] since TSS at CWTP was 144 ± 112 mg/L). Supernatant TSS ranged from 14-113 mg/L (excluding S12 during 1.4-d HRT), and there was not a clear relationship with SRT or HRT (Figure 5-14). Values were lower than those obtained by Travieso et al. (2006) (i.e., M=136 mg/L supernatant TSS) using microbial biomass to treat 451 mg/L tCOD wastewater with 4-d HRT. The microbial SBRs failed to consistently meet the 50-mg/L TSS MCL for wastewater discharge (per CRC051724) under any retention time combination investigated (Figure 5-14). CWTP also periodically has difficulty with (P6) effluent from the oxidation ponds (i.e., 36 ± 22 mg/L TSS) meeting the requirement under its current operating regime. Other harvesting methods such as filtration or DAF might be more effective at reducing supernatant TSS, but are also more expensive. Some sustainable solutions include recycling the C-depleted effluents and adding waste CO_2 for secondary nutrient recovery (Benemann 1997; Heubeck et al. 2007) or reusing effluents in irrigation applications following disinfection (Gray 2004).

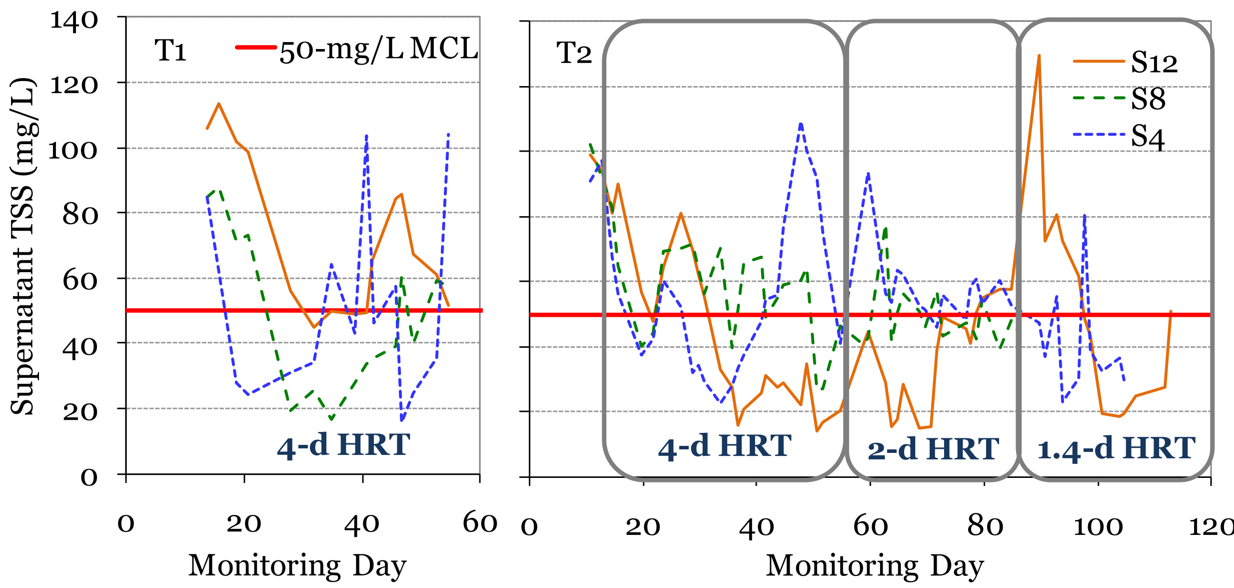

Figure 5-14. Supernatant TSS of Microbial SBRs Operated at Different SRTs and HRTs.

5.7 Summary

In summary, the following trends were elucidated using laboratory-scale microbial cultures fed with primary wastewater and operated at various retention times:

- Growth rate modelling indicated that bacterial productivity was predominantly C limited

at 4-d HRT and DO limited at 2- and 1.4-d HRTs. Meanwhile, microalgal productivity was probably light limited for all conditions.

- Total productivity ranged from 7.7-31.2 $g/m^2/d$ and was greatest on average at 21.6 $g/m^2/d$ for 2-d HRT and 12-d SRT. Some solids washout occurred at a shorter retention time indicating that 2-d HRT may be near the maximum loading limit for these systems.
- Settleability ranged from 36-98% and was generally greater and more consistent at longer SRT and shorter HRT. Solids recycling improved the performance to a minimum of 68% settleability.
- Settleability was sensitive to sedimentation duration and sedimentation container, so comparing actual and estimated measures is worthwhile to reliably indicate harvestable yield. Results indicated that sedimentation time could be reduced to 30 min with comparable performance.
- Settleable productivity indicated the quantity potentially harvestable following sedimentation and decantation. Settleable productivity was greatest at 20.6 $g/m^2/d$ for 2-d HRT and 12-d SRT.
- Soluble COD of primary wastewater was 162 ± 44 mg/L. Generally, COD removal increased from 63 to 77% as HRT and SRT increased. Culture operation at 4-d HRT with biomass recycling best met compliance requirements.
- Culture supernatants did not consistently meet the 50-mg/L discharge limit for suspended solids indicating a need for effluent reuse and/or secondary nutrient recovery.

These experiments contributed to understanding the effects of retention times (SRT and HRT) on productivity and settleability of microbial cultures. Longer SRT enhanced nutrient recovery and settleability of microbial cultures while shorter HRT enhanced productivity except when washout occurred. Meanwhile, DO production by photoautotrophs for heterotrophs and consequent microalgal-bacterial symbiosis was sensitive to wastewater loading and light penetration. An absolute HRT can not be optimal at all times due to customary variation of wastewater. Biomass recycling and regular monitoring of onsite COD and DO to tightly manage nutrient loading and prevent growth limitations are recommended in order to optimise microbial biomass production from HRAPs.

Chapter 6: Biomass Composition and Bioenergy Implications

6.1 *Microbial Ecology and Composition*

This research investigated symbiotic microalgal-bacterial systems capable of treating municipal wastewater. Other organisms including archaeans, fungi, protozoa, and microscopic invertebrate animals (e.g., rotifers and nematodes) were also present in the cultures. Hence, the microbial communities were inherently complex due to the numerous species present at various trophic levels and the multiple metabolic strategies employed at any given time. Figure 6-1 simplifies the trophic hierarchy of a wastewater treatment system.

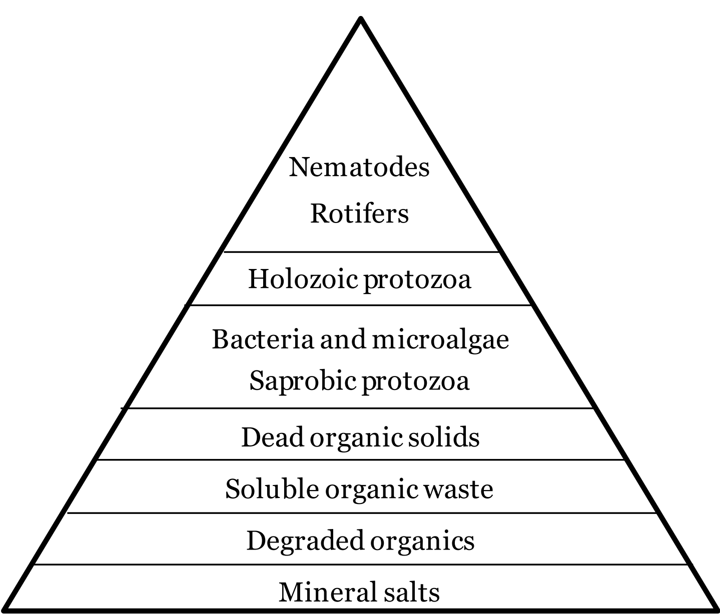

Figure 6-1. Food Pyramid of a Wastewater Treatment System. (Note: modified from Hawkes [1983].)

According to the principles of ecological engineering, cultures self-organise from their native inoculum(s) in response to the operational and environmental conditions imposed upon them (Mitsch and Jorgensen 2003). This strategy naturally selects for the best-adapted species to prevail under the given conditions with every specific operating regime sustaining a unique microbial community. In contrast to conventional engineering where ecosystems are often forcefully designed and their success measured by survival of specific organisms (e.g., trying to maintain a unialgal culture in an outdoor HRAP), self-organising systems are generally more resilient. Therefore, self-organisation theory was embraced for this research, which ultimately affected the biomass characteristics presented in this chapter.

6.1.1 Effects of Growth Conditions on Microalgal Community Composition

Regular taxonomic examinations using bright field light microscopy were made of samples cultured under various climatic and operational conditions to identify microalgae (and microfauna [Section 6.1.3]) potentially influencing the critical evaluation criteria (i.e., productivity, settleability, and composition) of the biomass. Generally, one slide was prepared of each culture at the start and end of experiments for examination. A total of 57 samples including 12 inocula and 45 cultures from this research were examined (Appendix A) resulting in the taxonomic diversity shown in Figure 6-2. Several commonly observed microbes are pictured in Figure 6-3. Some taxa present in low numbers may have been overlooked during these cursory examinations resulting in lower apparent diversity of samples. Identifying taxonomic trends was often difficult due to variation among replicates as well as the qualitative nature of the examinations. Therefore, taxonomic trends for specific conditions (i.e., climate, retention time, and feed wastewater) were generally based on species dominance, when apparent, and/or species occurrence.

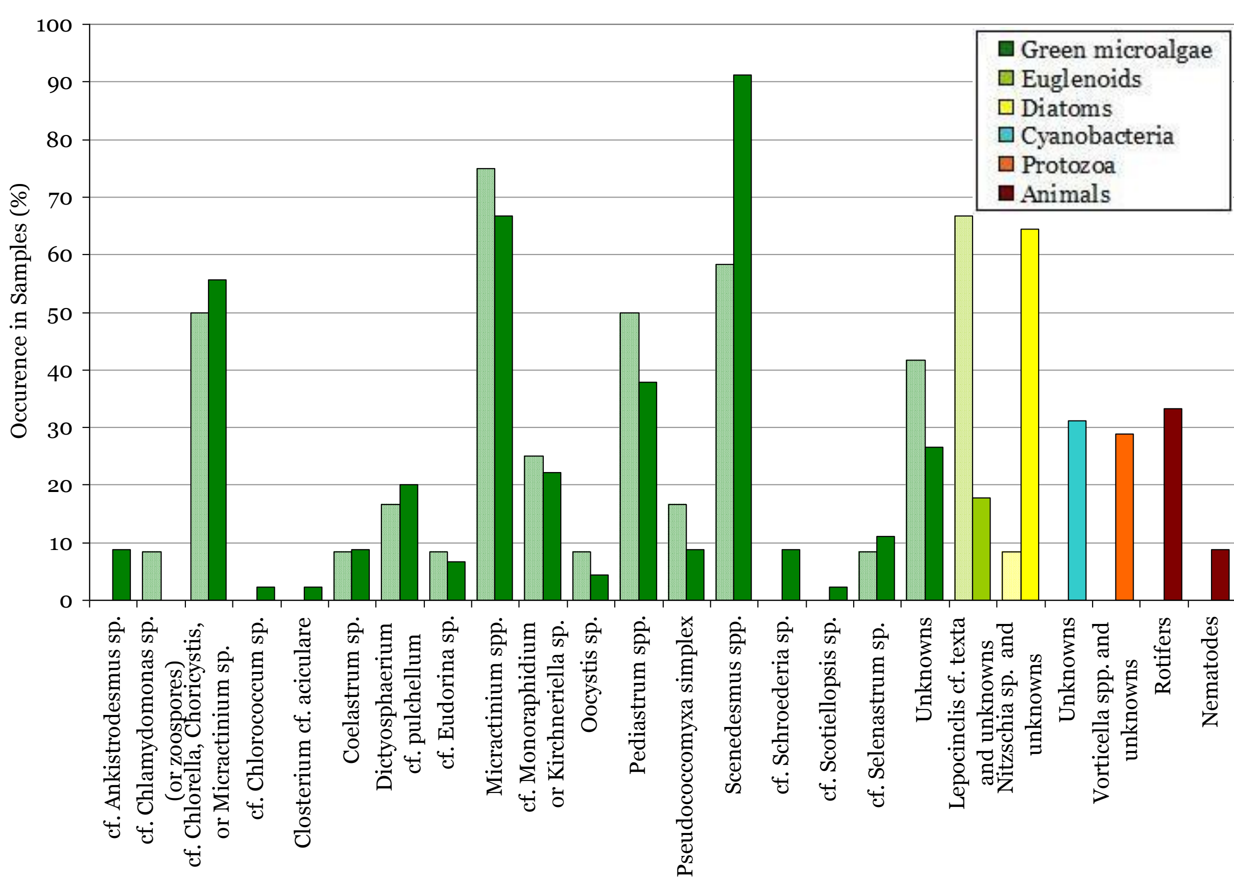

Figure 6-2. Species Occurrence in Microscopic Examinations of Inocula and Cultures. (Note: patterned columns represent oxidation pond inocula only and solid columns represent mixed laboratory cultures; cf.=comparable to; sp./spp.=one/multiple species.)

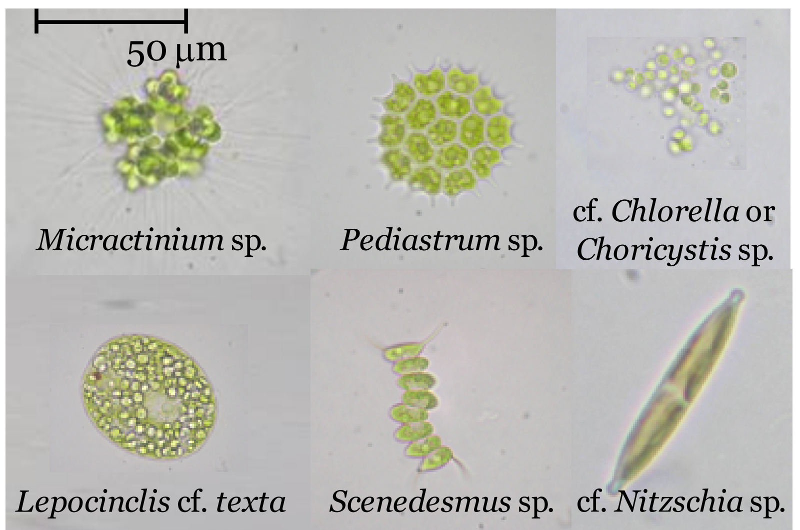

Figure 6-3. Photomicrographs of Microalgae Commonly Observed in Microbial Cultures.

P6 (wastewater and standardised) inoculum was usually dominated by green microalgae including *Micractinium* sp. and/or *Scenedesmus* spp. (i.e., 75% of samples), but occasionally by *Pediastrum* spp., cf. *Chlamydomonas* sp., or euglenoids (e.g., *Lepocinclis* cf. *texta*). Comparatively, the cultures promoted especially greater occurrences of *Scenedesmus* spp. (91 vs. 58%), diatoms (64 vs. 8%), Cyanobacteria (31 vs. 0%), and protozoa and animals (< 33 vs. 0%) (Section 6.1.3), but less euglenoids (18 vs. 67%) than the oxidation ponds (Figure 6-2). *Scenedesmus* spp., *Micractinium* sp., and/or *Pediastrum* spp. generally continued to dominate cultures (i.e., 78% of samples). Typically also found in HRAPs, these are large and/or colonial species, so they are generally amenable to harvesting by sedimentation (Heubeck and Craggs 2007).

SBR filling strategies (e.g., continuous, fed-batch) and light regimes may also affect the dynamics of microbial communities. For instance, rapid fill systems starting with high nutrients at the beginning of RPs (e.g., Section 5.1) probably selected for floc-forming bacteria since they can outcompete filamentous bacteria under these conditions (Artan et al. 2005). Moreover, dark period anaerobiosis probably selected for low-DO tolerant bacteria, which have been associated with sludge bulking (Artan et al. 2005). Hence, complex and interacting factors likely influenced the microbial communities and, thus, the cultures' performance. However, only effects of climate, wastewater feed, and retention times were further discussed in this chapter as they constituted the foci of this research.

6.1.1.1 Random Effects on Cultures

Microalgal communities self-organised in response to their growth conditions. Generally, the same communities can develop for the same climatic and nutritional conditions. However, some differences in microalgal taxa and relative abundances were observed between replicates

probably due to natural divergence of the complex communities over time (Matheson et al. 2008) combined with customary variation of the feed water due to industrial trade wastes, precipitation, and other factors. For instance, replicate CSTRs (Section 3.2) had similar occurrence and dominance of microalgae (Appendix A), but it was noted that one reactor (R2) contained more *Scenedesmus* spp. at the end of the experiment.

6.1.1.2 Climatic Effects on Cultures

Microscopic observations were separated into the three different climatic categories to discern trends in the taxonomy. *Pediastrum* spp., *Coelastrum* sp., and Cyanobacteria were observed primarily in Ambient and Warm Studies' cultures (i.e., 16, 4, and 14/33 vs. 1, 0, and 0/12 Cold Studies' samples, respectively [Appendix A]), and they only dominated in Warm Studies' cultures (Table 6-1). These patterns suggested that these species may be less tolerant to cold climates than other green microalgae such as *Micractinium* sp. or *Scenedesmus* spp., which could dominate under any condition (Table 6-1). Indeed, some Cyanobacteria prefer higher temperatures than green microalgae (McQueen and Lean 1987; Smith et al. 1987). In contrast, *Pseudococcomyxa simplex* was observed exclusively in Cold Studies' cultures (i.e., 4/12 samples [Appendix A]), which indicated that it may be more competitive in colder climates. Other species such as cf. *Ankistrodesmus* sp., cf. *Schroederia* sp., and cf. *Eudorina* sp. were observed strictly in Ambient Studies' cultures (Appendix A), which suggested that they may be most advantaged at warm temperature and low radiation (i.e., 19-28 °C and 7.3-9.7 MJ/m²/d). *Micractinium* sp. and *Scenedesmus* spp. were found under all climatic conditions trialled indicating that these species are likely to thrive year-round in outdoor HRAPs in Christchurch, NZ, as observed by others (Heubeck and Craggs 2007).

Table 6-1. Distribution of Microscopic Examinations of Cultures and Observed Dominants Grouped by Climatic Condition.

Thesis Section and Sources	Ambient Climate 19-28°C 7.3-9.7 MJ/m²/d	Warm Climate 16-26°C 21.3 MJ/m²/d	Cold Climate 8-18°C 6.2 MJ/m²/d
3.1: C1, C6, C6F Cultures	3 (X)		
3.2: R1, R2 Cultures	4 (E,T)		
3.3: SBR1/2 Cultures	4 (D,M,P,T)		
4.1: CO, SE, PE, AP Cultures		12 (C,M,P,T,U)	12 (F,M,O,T)
5.1: S4, S8, S12 Cultures		10 (C,D,M,T,U)	
Total Observations	**11/45**	**22/45**	**12/45**

C=Cyanobacteria; D=diatom; E=cf. *Selenastrum* sp.; F=*Dictyosphaerium* cf. *pulchellum;* M=*Micractinium* spp.; P=*Pediastrum* spp.; O=*Pseudococcomyxa simplex*; T=*Scenedesmus* spp.; U=unknown Chlorophyte; X=not apparent.

6.1.1.3 Wastewater Effects on Cultures

Microscopic observations were separated into the four different wastewater sources to discern trends in the taxonomy. Tap water (CO) cultures usually contained *Scenedesmus* spp. (i.e., 5/6

samples), but dominance varied across these cultures and was not always evident (Appendix A). Cyanobacteria were most often found in CO compared to other cultures (i.e., 50 vs. 17-44%, respectively) probably because some species can fix N_2 (and CO_2) from the air, which would have been highly advantageous in this nutrient-sparse condition. Secondary wastewater (SE) cultures were dominated by *Micractinium* sp. and/or *Scenedesmus* spp. (Table 6-2) while *Micractinium* sp. were only found in 17-75% of other cultures, which possibly indicated a lower CO_2 minimum or higher pH tolerance (i.e., M=8.3-10.2 SU for SE vs. M=7.2-8.5 SU for PE-AP [Section 4.2.2]) for growth of these species. Primary wastewater (PE) cultures supported the greatest diversity of microalgal genera on average (i.e., 7/sample vs. 5/sample for SE and AS-inoculated cultures and 4/sample for CO cultures [Appendix A]) probably due to more favourable growth conditions. For instance, diversity may have been enhanced in PE from comparatively lower DO and pH (with greater CO_2 availability) than in SE, and from greater light penetration than in AS-inoculated cultures. PE cultures consistently contained *Scenedesmus* spp., and they were usually dominated by *Scenedesmus* spp. and/or *Micractinium* sp. although several samples were abundant in *Dictyosphaerium* cf. *pulchellum*, *Pediastrum* spp., cf. *Selenastrum* sp., and/or *Pseudococcomyxa simplex* (Table 6-2). Moreover, cf. *Ankistrodesmus* sp., *Oocystis* sp., cf. *Schroederia* sp., and cf. *Selenastrum* sp. (i.e., 4, 2, 4, and 5/12 samples, respectively) were found exclusively in PE cultures (Appendix A). Eisenberg et al. (1981) also found that HRAPs treating primary wastewater were dominated by *Micractinium* spp., which indicated that PE cultures were ecologically comparable to outdoor systems.

Table 6-2. Distribution of Microscopic Examinations of Cultures and Observed Dominants Grouped by Operating Condition.

Thesis Section and Sources	Tap water	Secondary wastewater	Primary wastewater	AS inoculum + primary wastewater	
	H8 S8-40	H8 S8-40	H7-9 S8-40	H8-9 S8-80	H1.4-4 S4-12
3.2: R1, R2 Cultures			4 (E,T)		
3.3: SBR1/2 Cultures			2 (M,P,T)	2 (D,P)	
4.1: CO, SE, PE, AP Cultures	6 (C,T,U)	6 (M,T)	6 (F,M,O,P,T)	6 (F,M,P,T)	
5.1: S4 Cultures					3 (D,M,T)
5.1: S8 Cultures					3 (C,D,M,T,U)
5.1: S12 Cultures					4 (C,D,M,T)
Total Observations	**6/42**	**6/42**	**12/42**	**8/42**	**10/42**

Note: H=HRT(d); S=SRT(d); C=Cyanobacteria; D=diatom; E=cf. *Selenastrum* sp.; F=*Dictyosphaerium* cf. *pulchellum;* M=*Micractinium* spp.; P=*Pediastrum* spp.; O=*Pseudococcomyxa simplex*; T=*Scenedesmus* spp.; U=unknown Chlorophyte.

Inoculation of cultures with AS affected the microalgal communities' development. For instance, approximately 3 weeks following (12% v/v) AS addition to SBR1/2, community structure had changed from dispersed to flocculated microbes (Figure 6-4), productivity had halted (i.e., TSS was degrading), and diversity had decreased from 7 to 3 genera/sample (Appendix A) probably

due to ammonia toxicity, higher organic loading, and shading (Section 3.3). Only diatoms, *Pediastrum* spp., and unknowns (i.e., cf. *Chlorella* sp. and/or *Choricystis* sp.) remained in these (overloaded) reactors. *Chlorella*, *Nitzschia* (diatoms), and *Scenedesmus* are among the most tolerant genera for organic pollution (Palmer 1969; de Godos et al. 2009). Sampling results by Christchurch City Council also suggested that *Chlorella* sp. and/or *Choricystis* sp. were less sensitive to wastewater quality than other taxa in P6 (Novis 2007). Cultures inoculated with more conservative amounts of AS (2% v/v) and fed primary wastewater were usually dominated by *Scenedesmus* spp., *Micractinium* sp., and/or diatoms, but some samples were also abundant in *Pediastrum* spp. or Cyanobacteria (Table 6-2). Diatoms were most often found in AS-inoculated cultures compared to other cultures (i.e., 89 vs. 17-67%, respectively [Appendix A]) probably because they grow better at lower light intensities (i.e., limited to 50% of μ_m at a lower [K_s] value) (Richardson et al. 1983). This physiological attribute gave diatoms a competitive advantage over green microalgae in light-limited environments, but disadvantaged them in greater light. This observation was also made by Tarlan et al. (2002), who found that diatoms outnumbered green microalgae at lower light intensity and higher COD loading of paper industry wastewater.

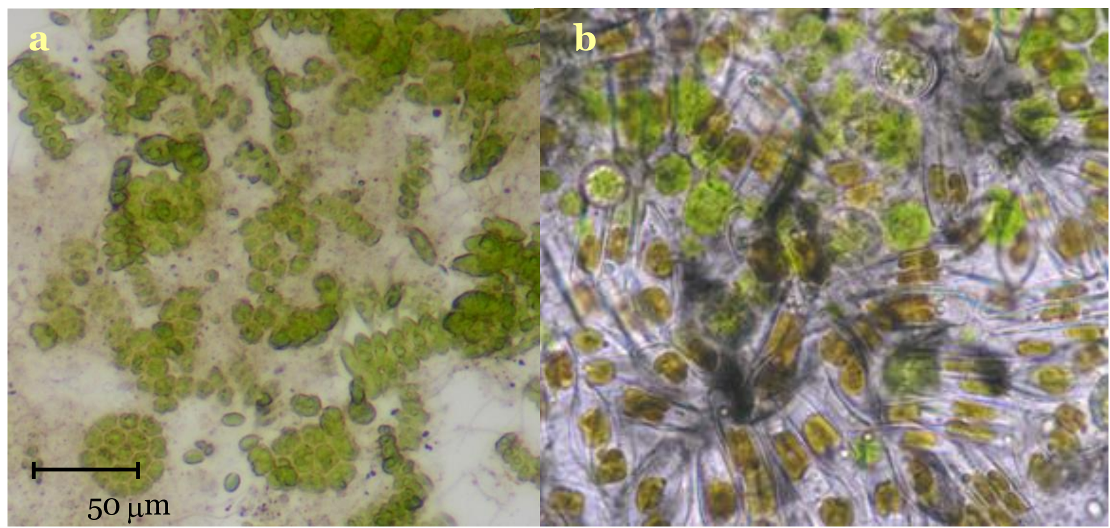

Figure 6-4. Microbial Community Differences between Primary Wastewater Cultures a) Lightly-Loaded and b) Overloaded with AS Inoculum. (Note: [a] mostly *Scenedesmus* sp. and *Pediastrum* sp. and [b] mostly diatoms and *Pediastrum* sp.)

6.1.1.4 Retention Time Effects on Cultures

The effects of retention times on the microalgal communities were also examined. Broadly, two conditions could be compared for AS-inoculated, primary wastewater cultures: 1) long retention times with 8- to 9-d HRT and 8- to 80-d SRT and 2) short retention times with 1.4- to 4-d HRT and 4- to 12-d SRT (Table 6-2). Plausibly, long SRT selected for settleable microbes that were efficient under low light, while short SRT selected for microbes with higher maximum growth

rates. Additionally, long HRT favoured aerobic species while short HRT favoured species efficient under low oxygen (Section 5.2.2).

Scenedesmus spp. and *Micractinium* sp. were abundant for both retention time conditions (Table 6-2). Larger species such as *Pediastrum* spp. were more often found at long compared to short retention times (i.e., 50 vs. 10% [Appendix A]) probably owing to slower maximum growth rates. However, *Micractinium* sp., Cyanobacteria, and diatoms were more prevalent at short compared to long retention times (i.e., 90 vs. 63%, 80 vs. 19%, and 100 vs. 53%, respectively [Appendix A and Table 6-2]). Among (10) short retention time samples, the only discernable differences for SRT (partly due to the small sample sizes and variation between replicates) were that 12-d SRT cultures (S12) were always dominated by *Scenedesmus* spp., and that *Micractinium* sp. became more abundant as SRT decreased. Additionally, among the (4) samples from < 4-d HRT cultures, all of them were dominated by *Scenedesmus* spp. (Appendix A). In contrast, Cromar and Fallowfield (1997) observed less *Scenedesmus* sp. and more Cyanobacteria as COD loading increased from 10 to 60 $g/m^2/d$.

Greatest productivity and settleability were achieved while operating at short retention times. A 2-d HRT and 12-d SRT culture dominated by *Scenedesmus* spp. optimised settleable TSS productivity of microbial biomass. Microfauna including algivores and bacterivores were absent from this culture (Section 6.1.3), which would have also contributed to the increase in productivity from 4- to 2-d HRT.

6.1.2 Effects of Growth Conditions on Bacterial Community Composition

Bacterial community composition of select cultures from this research was determined at the University of Auckland using the 16S rRNA strategy described by Hugenholtz (2002). (Although photosynthetic Cyanobacteria were considered to be microalgae throughout this research, they are technically bacteria, so they were also analysed by this method.) Gene clone libraries from settled and supernatant biomass samples were sequenced and identified at a 95% confidence level using the Classifier program from the RDP (Appendix A). It was hypothesised that the biomass fractions would exhibit different bacterial community compositions, and by comparing them, bacteria potentially impacting the critical evaluation criteria could be identified. Overall, similar taxa were found for these cultures and wastewater treatment applications in the literature (Du et al. 2008; Wang et al. 2011).

Climate appeared to have a greater effect on the bacterial communities of SE, PE, and AP cultures than the particular feed water. Warm Studies' settled biomass contained much more Cyanobacteria (12-34%) and Firmicutes (3-10%) compared to Cold Studies' settled biomass (0-1% for both) (Figure 6-5), which suggested that these taxa grow better in warm climates (McQueen and Lean 1987; Smith et al. 1987). The absence of Cyanobacteria in Cold Studies'

bacterial gene libraries agreed with the microscopic results (Section 6.1.1.2). In contrast, Cold Studies' settled biomass contained more Bacteriodetes (23-27%) compared to Warm Studies' settled biomass (1-6%), so they may have a competitive advantage in colder climates. Proteobacteria ranged from 50-70% for all settled biomass samples, and it was comprised mostly of β- and γ-Proteobacteria for the Cold Studies, but with a greater portion of α-Proteobacteria for the Warm Studies (Figure 6-5). These settled biomass findings were comparable to those of Morgan-Sagastume et al. (2008) who examined AS. Within the Cold Studies' supernatant biomass, β-Proteobacteria (38-83%) dominated, and abundances of γ-Proteobacteria were somewhat lower compared to the settled biomass.

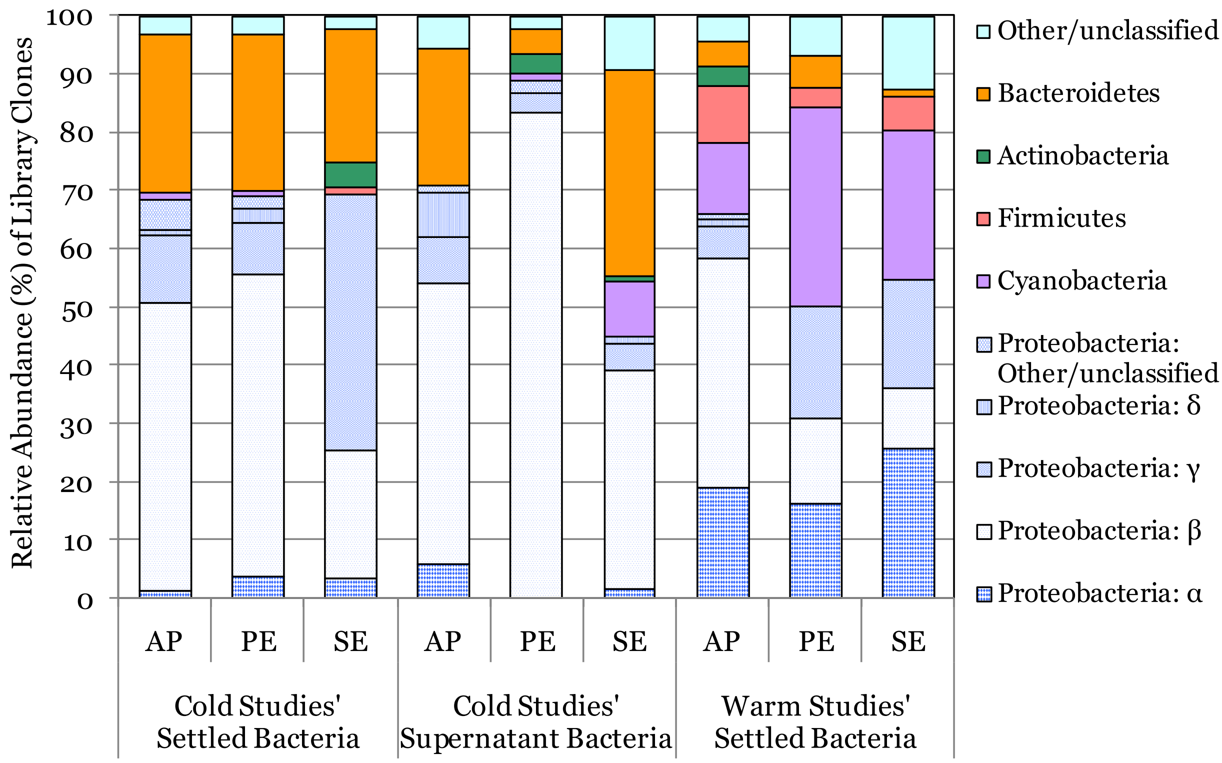

Figure 6-5. Bacterial Community Composition of Settled and Supernatant Samples from Municipal Feed Water Selection Experiments. (Note: sequences not aligning to known taxa within the 95% confidence limit indicated as other/unclassified; patterns of same colour represent classes of common phylum; labels refer to cultures as described in Section 4.1.)

The AP, PE, and SE results (Figure 6-5) differed substantially from those obtained for SBR1 (Figure 6-6) probably due to AS overloading. The bacterial community within the AS-inoculated, settled biomass of SBR1 was dominated by Firmicutes (45%) and Proteobacteria (39%) phyla (Figure 6-6). Notably, Clostridia, an obligately anaerobic class of Firmicutes, accounted for 36% and *Thiothrix*, a sulphur oxidising genus of class γ-Proteobacteria, comprised 18% of library clones. These results supported the observation of anaerobic conditions following AS addition as a result of reactor overloading. Supernatant from the same SBR1 culture contained less Firmicutes (26%) and more Proteobacteria (68%) than the settled

biomass, which indicated that Firmicutes were more settleable and probably highly enmeshed within the microbial flocs compared to the more ubiquitous Proteobacteria. β-Proteobacteria (60%) dominated the supernatant biomass library of SBR1 compared to only 10% in settled biomass (Figure 6-6). Similarly, β-Proteobacteria (54-83%) also dominated the Cold Studies' supernatant biomass of PE and AP (Figure 6-5). Morgan-Sagastume et al. (2008), however, found that Firmicutes and Planctomycetes were more abundant in supernatants from an AS process—possibly due to site-specific differences including minimal (if any) microalgae possibly affecting community dynamics. No other taxonomic trends regarding settleability or productivity were discernable among the gene clone libraries.

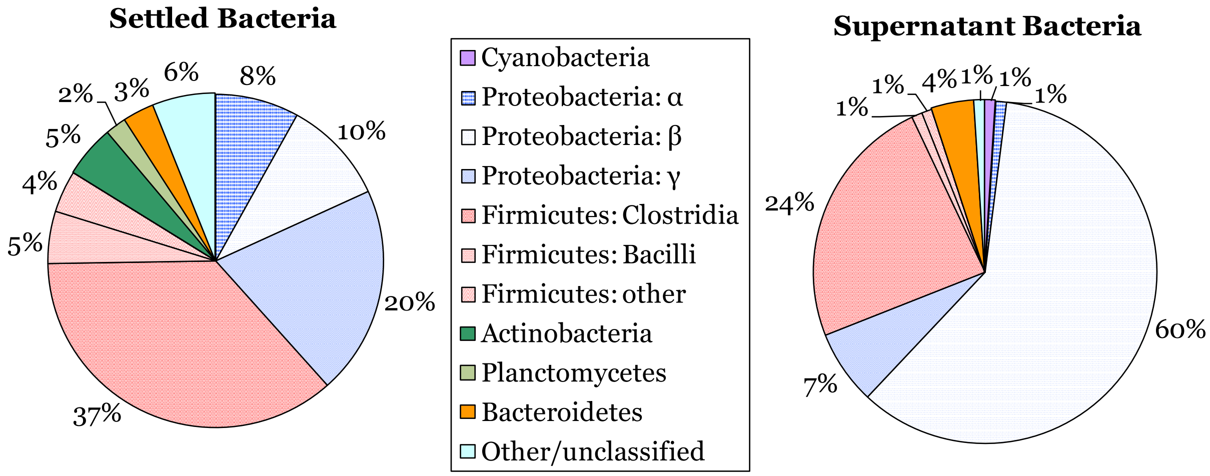

Figure 6-6. Bacterial Community Composition of Settled and Supernatant Samples from Culture Overloaded with Activated Sludge. (Note: percentage of library clone sequences aligning to each taxonomic group indicated; patterns of same colour represent classes of a common phylum.)

6.1.3 Effects of Growth Conditions on Microfaunal Community Composition

Microfauna including animals (e.g., rotifers, nematodes) and protozoa (e.g., ciliates such as *Vorticella* spp.) were also present in the microbial cultures although not observed in inocula (Figure 6-2). Microfauna can consume up to 41% of microalgal (and bacterial) biomass in one day, but microalgae > 50 µm are generally safe due to mouth size restrictions of algivores (Graham et al. 2009). Optimum growing conditions for a rotifer, *Brachionis rubens*, were summarised by Schlüter et al. (1987) as 15-25°C, pH of 6.5-8.5 SU, and retention times > 2 d to prevent washout. These criteria support observations of this research since rotifers (and other microfauna) were rarely found in Cold Studies (8-18°C) (i.e., 2/12 samples), secondary wastewater (*M*=8.3-10.2 SU) (i.e., 2/6 samples), and short retention time cultures (i.e., 0/4 samples at < 4-d HRT).

Microfauna probably were not able to establish themselves at short (< 4-d) HRT because of slower maximum growth rates, and this would have also contributed to the increase in TSS productivity as HRT decreased from 8 to 1.4 d. In addition, *Vorticella* spp. and other algivorous ciliates grow slower than microalgae in colder temperatures (Graham et al. 2009), which explained their absence from Cold Studies cultures. In contrast, microfauna were observed in up to 59% of Warm Studies (i.e., 13/22 samples) and 40% of PE and AS-inoculated cultures (i.e., 12/30 samples)—all of which had long (≥ 4-d) HRT. Nematodes were absent and other microfauna were rare in CO (i.e., 1/6 samples contained rotifers) and SE cultures (i.e., 2/6 samples contained rotifers and ciliates) probably because they are at the top of the food chain (Figure 6-1), and limited food existed within these compared to other cultures.

Algivorous and bacterivorous microfauna are often beneficial in reducing supernatant TSS through consumption of solitary, suspended prey leading to improved settleability (Gray 2004). However, mean settleability was 69-87% with microfauna at 4- to 8-d HRT and 76-95% without microfauna at ≤ 2-d HRT indicating that they were not a prevailing factor. In addition, algivores may avoid certain microalgal species due to food quality or ease of consumption. For example, rotifers cannot ingest *Micractinium pusillum* with long spines due to their size (Schlüter et al. 1987). Despite this, the only apparent taxonomic difference observed between cultures with and without microfauna suggesting selective feeding, was a greater presence of unknown green, spherical microalgae (i.e., cf. *Chlorella* sp.) in cultures without microfauna (i.e., 24/29 vs. 1/16 samples containing microfauna and unknown spheres).

6.2 Chemical Composition

Microbial biomass is principally composed of lipids, proteins, and carbohydrates. Small quantities of minerals, nucleic acids, sterols, pigments, and vitamins are also present. The relative proportions of these constituents can be highly affected by environmental conditions (Becker 1994; Richmond 2004). Biomass composition influences its combustion and ash production (Obernberger et al. 1997). Several key parameters were characterised below to determine the biomass' quality primarily for biofuel applications, but also for other uses such as fertilisers.

6.2.1 Organic and Inorganic Matter

A strong positive correlation existed between TSS and VSS ($r[37]=1.00$) throughout this research, and analysis of VSS confirmed that TSS was predominantly organic (M=90, SD=3.4%) (Figure 6-7). Highly organic feedstock is very combustible and contains fewer inorganic compounds, which have been shown to impact combustion (Obernberger et al. 1997; 2006). Hence, solids were generally referred to as 'biomass,' and no calculations were made to specify organic fractions throughout this thesis although some inorganic matter existed (M=10, SD=3.4%). Therefore, productivity values represented all filterable solids within the reactors

including microbes, sewage organics, and inorganic matter. In comparison, the inorganic content of the microbial biomass was similar to AS (10%) (Orhon et al. 2009), but higher than wood fuels (0.3-5%) (Obernberger et al. 2006), indicating less efficient combustion.

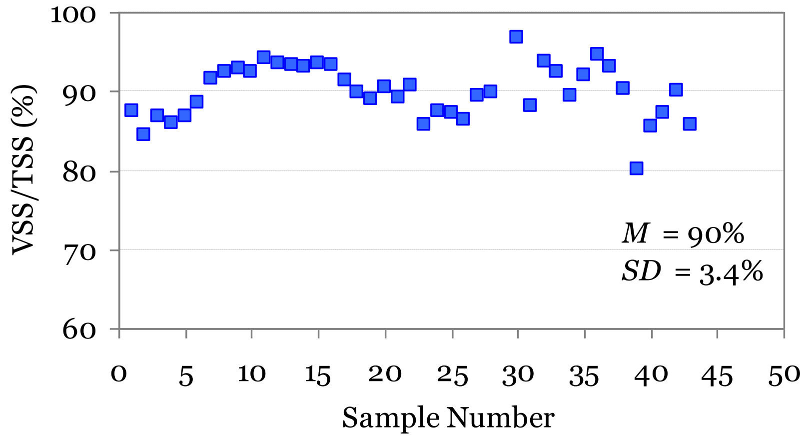

Figure 6-7. Organic Contents of Microbial Cultures.

Areal and volumetric TSS productivities obtained during this research for stable cultures are summarised in Table 6-3. Maximum total TSS productivity obtained using 4-d SRT and 1.4-d HRT was similar to that presented in the literature (Becker 1994). Mean and maximum settleable productivities (i.e., 20.6-25.2 $g/m^2/d$) were optimised at 12-d SRT and 2-d HRT indicating that these were the most favourable conditions examined for productivity and settleability.

Table 6-3. TSS Productivities of Stable Microbial Cultures.

Parameter	Areal Value ($g/m^2/d$)	Volumetric Value ($g/m^3/d$)	Growth Conditions
Maximum Total TSS Productivity	31.2	98.1	Warm climate; H1.4; S4 (Section 5.1)
Mean Total TSS Productivity	21.6	68.0	Warm climate; H2; S12 (Section 5.1)
Maximum Settleable TSS Productivity	25.2	79.3	Warm climate; H2; S12 (Section 5.1)
Mean Settleable TSS Productivity	20.6	64.8	Warm climate; H2; S12 (Section 5.1)

Note: conditions as referred to in thesis sections listed for each wastewater type with retention times (H=HRT[d]; S=SRT[d]).

6.2.2 Chl a to Estimate Microalgal (and Other) TSS Contents

Chl *a* results directly indicated the presence of microalgae in microbial biomass (i.e., TSS) and photosynthetic oxygenation capacity of cultures (Section 5.2.2), but uncertainty in absolute values existed since Chl *a* content of microalgae can vary from 1-2% and can be biased by culture conditions (Section 2.4.6). Non-microalgal (assumed predominantly bacterial) TSS was calculated by difference of total TSS and microalgal TSS, so it was also impacted by any Chl *a* biases. For these reasons, Chl *a* results more appropriately served as a culture dynamics indicator rather than an absolute fractionalisation tool.

The validity of Chl *a* results was questioned following examination of trends from one experiment (despite laboratory accreditation for the analytical method). Specifically, two replicate operational periods (T1 days 0-70 and T2 days 0-55 during H4 [Section 5.1]) had substantially different results (i.e., T1 days 0-47: *M*=0.9, *SD*=0.4; T2 days 0-55: *M*=2.3, *SD*=0.7 mg/L Chl *a*) (Figure 6-8a,b). While this in itself would not necessarily cause concern, it was also noticed that values for one replicate (T1) were substantially different depending on laboratory batch (i.e., B1 days 0-47: *M*=0.9, *SD*=0.4; B2 days 48-70: *M*=3.1, *SD*=1.2 mg/L Chl *a*) despite consistent operational conditions for the reactors. Chl *a* was also divided by TSS to normalise values across cultures containing varying amounts of solids (Figure 6-8c,d). Differences between batches were further emphasised, and values above 15 mg Chl *a*/g TSS during T1 days 48-70 were revealed (Figure 6-8c). Based on the *a priori* assumption of 1.5% Chl *a* in microalgae, this indicated that nearly all solids (and even over 100%) were microalgae. In actuality, this could not be accurate since visual observations confirmed the presence of non-microalgal solids such as bacteria, microfauna, and sediment. Upon review by the laboratory, no instrumentation, analytical, or data entry errors were found. While it was possible that Chl *a* represented a smaller portion of microalgae than assumed (e.g., < 1.5%), it still seemed likely from the batch and replicate differences that some of the data were erroneous. In light of this investigation, T1's Chl *a* data were discarded from further discussion in this thesis so that a comparable basis existed between the different HRTs for T2.

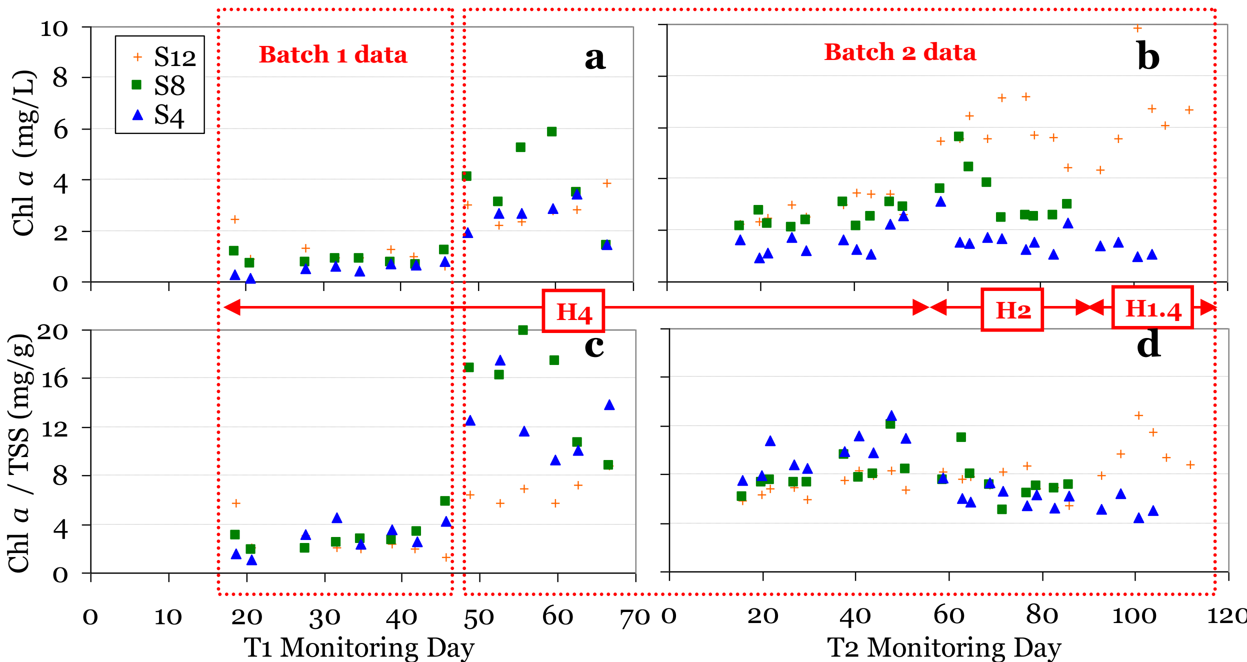

Figure 6-8. Chl *a* from Two Different Sample Batches (B1 and B2) and Replicates (T1 and T2) Based on Raw Laboratory (a, b) and TSS-Normalised (c, d) Results.

The remainder of the Chl *a* data were normalised to estimate microalgal portions of solids produced during this research and examined for effects of climate, feed water, and retention

times (Table 6-4). Microalgal portions were greatest at 73-85% for SE, PE, and AP during the Cold Studies (Figure 6-9). However, climate effects were not statistically significant for these cultures ($t[10]=2.02$, $p=0.07$) (Figure 6-9), although significance may improve with larger sample size. For example, Ip et al. (1982) also found that low temperature enhanced microalgal growth because of increased CO_2 solubility. Insignificant difference also existed for wastewater feed condition in the range of 54-85% for SE, PE, and AP cultures ($F[2,12]=0.12$, $p=0.89$) (Figure 6-9), while CO only had a microalgal content of 14% due to nutrient limitation.

Table 6-4. Chl *a* Contents and Microalgal Portions of Reactor Solids.

Thesis Section and Conditions	SBR	Sample Count	Chl *a*/TSS (mg/g)			Microalgal Portion (%)[a]		
			M	±	***SD***	***M***	±	***SD***
3.3: Ambient Climate, H9, S8-80	SBR1/2 (without AS)	6[b]	8.3	±	1.3	55	±	9
	SBR1/2 (with AS)	6[b]	1.3	±	0.1	9	±	1
4.1: Cold Climate, H8, S8-40	CO	2[c]	2.1	±	0.3	14	±	2
	SE	2[c]	10.9	±	4.0	73	±	27
	PE	2[c]	12.7	±	4.2	85	±	28
	AP	2[c]	10.9	±	1.9	73	±	13
4.1: Warm Climate, H8, S8-40	CO	3[c]	2.1	±	0.4	14	±	3
	SE	3[c]	8.1	±	3.6	54	±	24
	PE	3[c]	8.4	±	3.0	56	±	20
	AP	3[c]	9.2	±	0.9	61	±	6
5.1: Warm Climate, H4, S4-12	S12	10[b]	7.1	±	0.9	47	±	6
	S8	10[b]	8.2	±	1.6	54	±	11
	S4	10[b]	9.8	±	1.7	66	±	11
5.1: Warm Climate, H2, S4-12	S12	8[b]	7.7	±	0.7	51	±	5
	S8	9[b]	7.4	±	1.6	49	±	11
	S4	8[b]	6.3	±	0.9	42	±	6
5.1: Warm Climate, H1.4, S4-12	S12*	6[b]	10.0	±	1.8	67	±	12
	S4	4[b]	5.3	±	0.8	35	±	5

Note: SBRs as referred to in thesis sections listed for each wastewater type and climate with retention times (H=HRT[d]; S=SRT[d]); (a) assuming 1.5% Chl *a* in microalgae; (b) measured over time; (c) measured once at end of replicate studies; *SRT not held constant due to solids washout.

Effects of retention times on microalgal contents were also examined, but could not be statistically analysed using *t*- or *F*- tests due to data dependence and absence of replicates. For stable cultures, S4 logically had the greatest microalgal content of 66% during H4 since it had the greatest light penetration (i.e., lowest TSS) permitting photosynthetic growth (Figure 6-10). The trend reversed during H2 as greater C loading increased heterotrophic metabolism, and any photosynthetic advantage of microalgae comparatively diminished. Bacteria may also reproduce faster than microalgae, so they could have better coped with the higher flowrate while microalgae were reduced to a minimum of 35% of the solids at shorter (< 4-d) HRT.

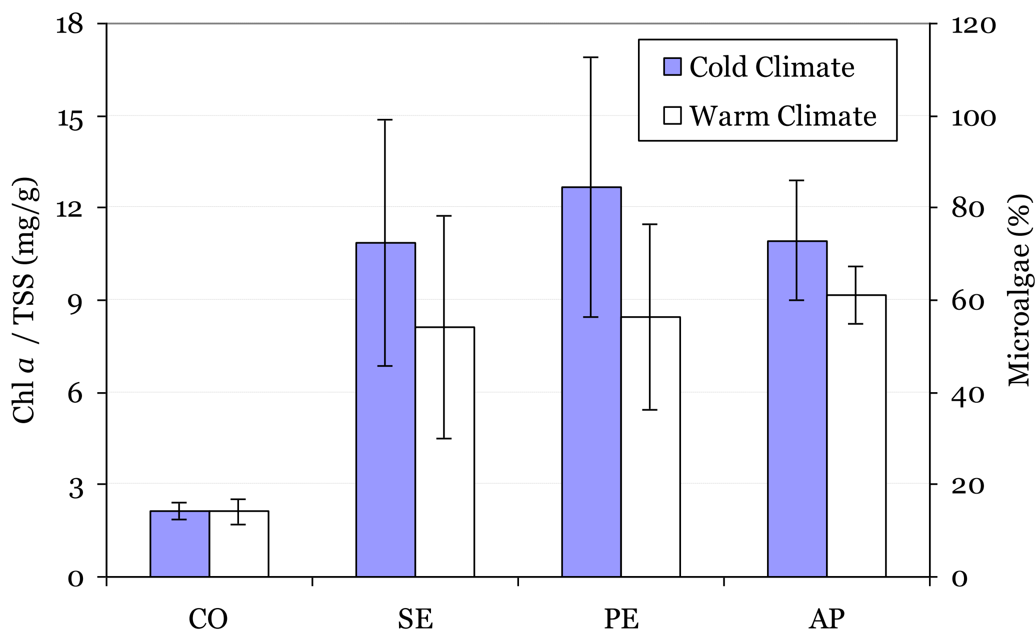

Figure 6-9. Effects of Climate and Feed Condition on Normalised Chl *a* Contents of Reactor Solids.

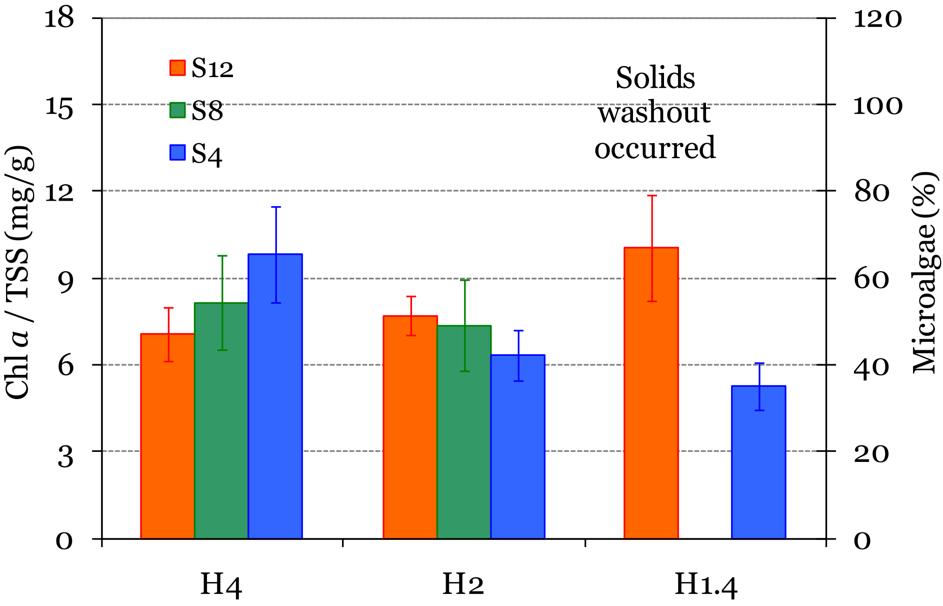

Figure 6-10. Effects of Retention Times on Normalised Chl *a* Contents of Reactor Solids.

Microbial cultures were sensitive to microalgal and bacterial proportions influencing microbial symbiosis. Wastewater treatment capacity and growth of microbial cultures were jeopardised following AS addition to SBR1/2 due to overloading, ammonia toxicity, and/or shading (Section 3.3.3.2). Culture inoculation with 2% AS provided a conservative amount of biomass for floc formation plus additional nutrients to AP and S4-S12 cultures. None of the cultures except SBR1/2 appeared to be anaerobic, but sufficient oxygenation by microalgae was questionable for

some cultures (i.e., S12 during H4 and S4-S12 during H2 and H1.4 [Section 5.3.3]). Cultures presumably found a symbiotic balance with a minimum 2-d HRT around 40-80% microalgal TSS, and the microalgal estimate agreed with others (Eisenberg 1981; Park and Craggs 2010).

6.2.3 Lipids

Lipid quantity and type have a significant influence on biodiesel quality (Knothe 2005) and nutrition (Becker 1994). Lipids are classified as non-polar (lipophilic) or polar (hydrophilic). All lipids contain non-polar fatty acid chains, and some also contain polar moieties (e.g., alcohols, sugars, etc.). The common categories of microalgal lipids are shown in Figure 6-11. As indicated, triglycerides (i.e., three fatty acids combined with glycerol) and hydrocarbons (i.e., lipids containing only C and H) are most suited for biodiesel. Microalgal lipids are usually predominantly triglycerides (i.e., 80-98%) (Becker 1994; Mata et al. 2010) although some species such as *Botryococcus braunii* have shown potential for pure hydrocarbons (Enssani 1989; Sawayama et al. 1992).

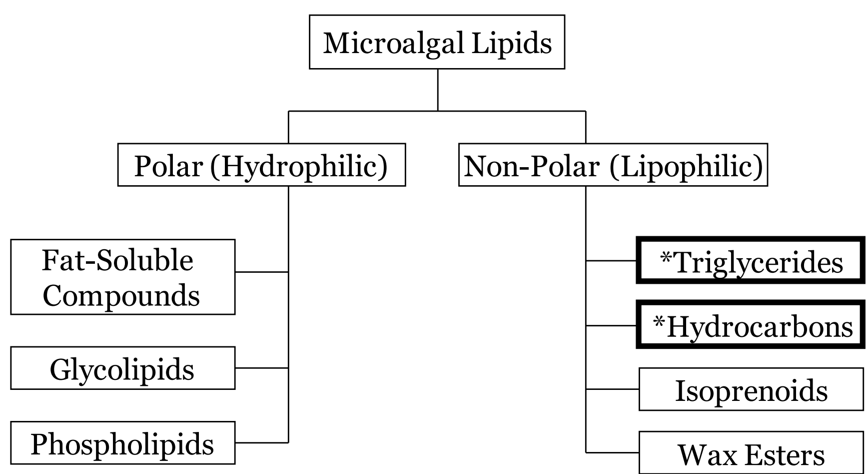

Figure 6-11. Categories of Microalgal Lipids.

(Note: *most suited for biodiesel; modified from Enssani [1989].)

Generally, fatty acids found in microalgae contain 12-22 C atoms, and they can be saturated (i.e., containing only single bonds and unable to add H [SFA]) or unsaturated (i.e., containing one [MUFA] or more [PUFA] double bonds and able to add H). Neither wastewater feed nor climate appeared to substantially affect the lipid profile since quantities of SFAs (1.9-3.1%), MUFAs (1.6-3.9%), and PUFAs (1.0-6.1%) were similar for all cultures (Figure 6-12). However, limited sample size (i.e., 1 sample/treatment/climate) prevented statistical analysis because biomass from replicate studies was combined to obtain sufficient quantity for laboratory testing. Shorter SFAs are desired for conversion into biodiesel as they have greater storage stability and are less likely to polymerise during combustion (Sheehan et al. 1998), even though they have poorer cold temperature properties (Knothe 2005; Sharma et al. 2008). Longer PUFAs are susceptible to oxidation during storage and gum and resin formation (Sheehan et al. 1998), but they have

nutritional importance for humans and aquaculture (Becker 1994). PUFAs were somewhat greater for all samples (i.e., 4-153% more) during the Cold compared to the Warm Studies which supported previous research showing that low water temperatures enhance PUFA production (Becker 1994; Blanchemain and Grizeau 1999). A larger portion of unidentified fats comprised the total lipid content of the Warm Studies (i.e., 0.1-1.9% vs. 0.0-0.4% for Cold Studies), which could impact the overall constituent proportions and abundances.

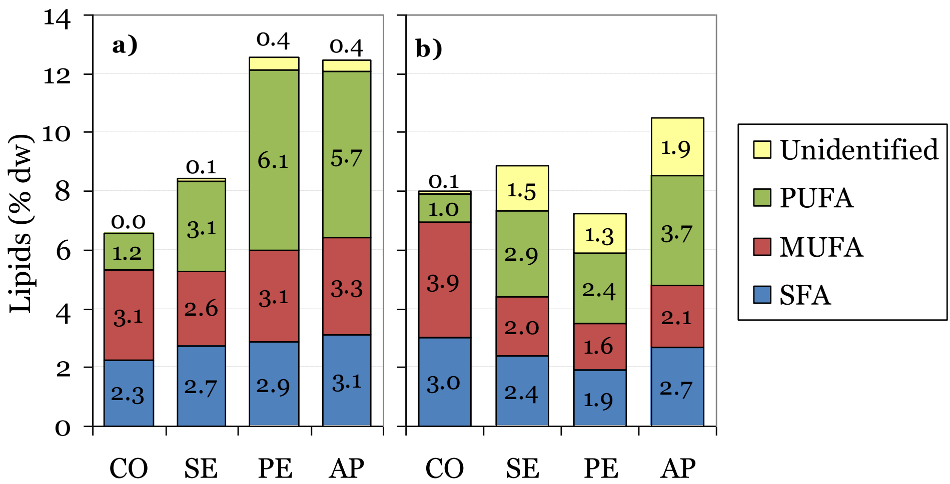

Figure 6-12. Microbial Biomass Lipids of a) Cold and b) Warm Studies' Cultures.

Effects of retention times on lipid quantities and profiles of AS-inoculated, primary wastewater cultures were not examined (primarily due to budgetary constraints). Total lipid values of AP cultures were already relatively low at 10.5-12.5%. It has been well-documented that low soluble nitrogen levels can stimulate lipid accumulation (Benemann and Oswald 1996; Sheehan et al. 1998). At higher flowrates during the retention time experiments compared to the feed water selection experiments, soluble nitrogen levels would have been higher after wastewater addition, so it was probable that lipid contents would be lower. Based on the bioenergy implications for low lipid content (Section 6.3.1), this biomass was not a sustainable source of lipids, so future testing was not warranted.

Overall, biomass contained 6.6-12.6% lipids (Figure 6-12). These values were low compared to pure microalgal cultures, which can attain up to 85% lipids (Richmond 2004), but were comparable to those obtained from transesterification of municipal sludge. Primary sludge reportedly contains up to 14.5% lipids whereas secondary (or activated) sludge contains 2.5-6.2% lipids (Dufreche et al. 2007; Mondala et al. 2009). To maximize lipids production, these results support the current strategy of screening and settling raw wastewater to obtain primary sludge (at 14.5% lipids), and then using primary wastewater to grow microbial biomass (at 7.2-12.6% lipids) to potentially optimise lipids production over conventional AS processes.

6.2.4 *Heat of Combustion*

Biomass HC is an important fuel property that indicates energy content and combustion efficiency. Mean biomass HC from PE and AS-inoculated cultures ranged from 22-26 MJ/kg (Appendix C). Biomass from CO and SE cultures was not able to be tested for HC due to the small overall sample size and overriding priority for lipids analysis. A maximum of 28-29 MJ/kg was reported for pure microalgal cultures containing lipid contents of 58-63% (Illman et al. 2000; Scragg et al. 2002). In general, lipid content correlates well with HC (Illman et al. 2000). Therefore, due to the lower lipid content of biomass from CO and SE cultures, they likely also had lower HC indicating less energy potential compared to biomass from PE and AS-inoculated cultures.

6.2.5 *TC, TKN, and TP*

Microalgae and bacteria generally require about 50% C, 6-12% N, and < 1% P by dry cell weight (Humenik and Hanna 1971; Brock 1981). These constituents were monitored because they have implications for biomass usage as biofuel and fertiliser (Sections 6.3 and 6.4). N content varies by species from 1 to > 10% (Richmond 2004) with green microalgae usually containing 5-10% (Becker 1994). N is a component of proteins, nucleic acids, and other cell constituents, and P is required for nucleic acids and phospholipids (Madigan et al. 2000). Linear regression of data from the retention time studies (i.e., means for each replicate; n=6) and controlled climate studies (i.e., run end values; n=20) indicated TC, TKN, and TP contents of approximately 45, 8, and 2%, respectively (according to slopes; Figure 6-13). These values are supported by strong coefficients of determination (R^2: 0.82-0.98; Figure 6-13) indicating consistent elemental biomass compositions across various cultures. N content was higher than that of wood and herbaceous biomass (< 4%) indicating potentially greater emissions of N_2 and environmentally harmful nitric oxides (NO_x) for microbial biomass compared to other fuels (Obernberger et al. 2006).

6.3 *Bioenergy Implications*

A wide range of biofuels can be produced from microbial biomass using various pathways (Figure 6-14). Brennan and Owende (2010) provided a comprehensive review of current technologies for microbial biomass conversion. Biomass dewatering and/or intensive drying is required for some processes to improve net energy output. Overnight sedimentation and primary dewatering of microbial biomass resulted in a solids' content of 1.6% (Section 3.2.4) indicating that secondary dewatering may be necessary for efficient biomass conversion. For example, anaerobic digestion tolerates much higher biomass moisture contents than combustion (i.e., 80-90% vs. < 50% moisture) (McKendry 2002) and may offer an energy- and cost-efficient processing strategy for biofuel.

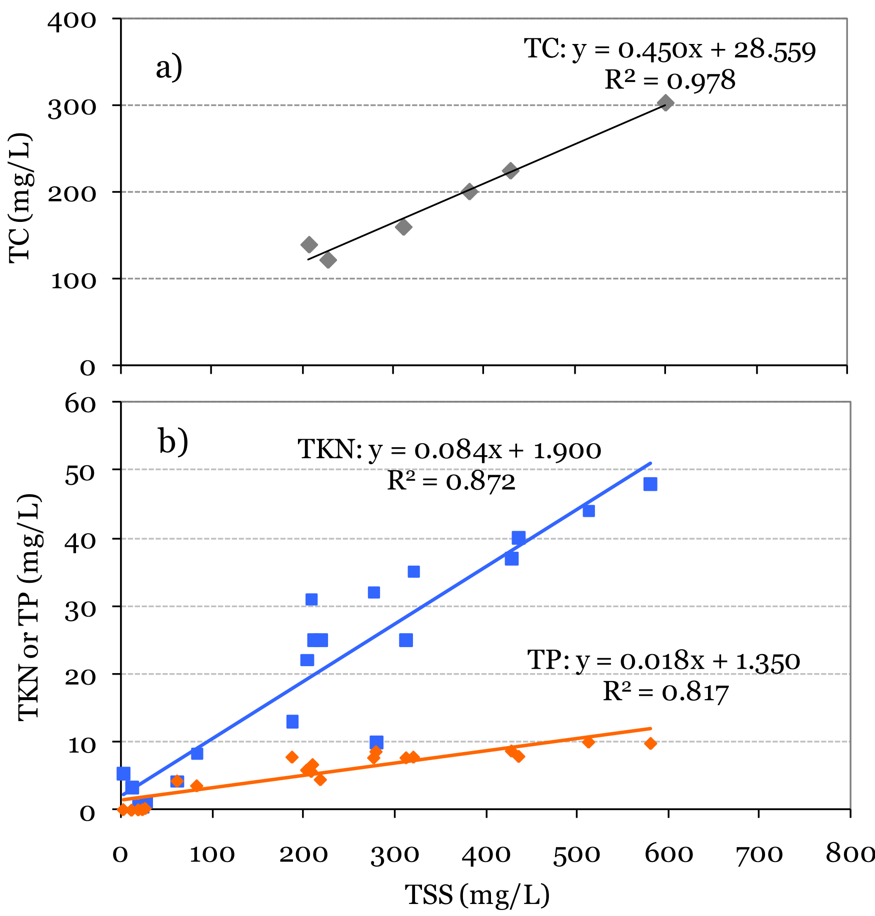

Figure 6-13. Linear Regression of TC, TKN, and TP Contents of Microbial Cultures.

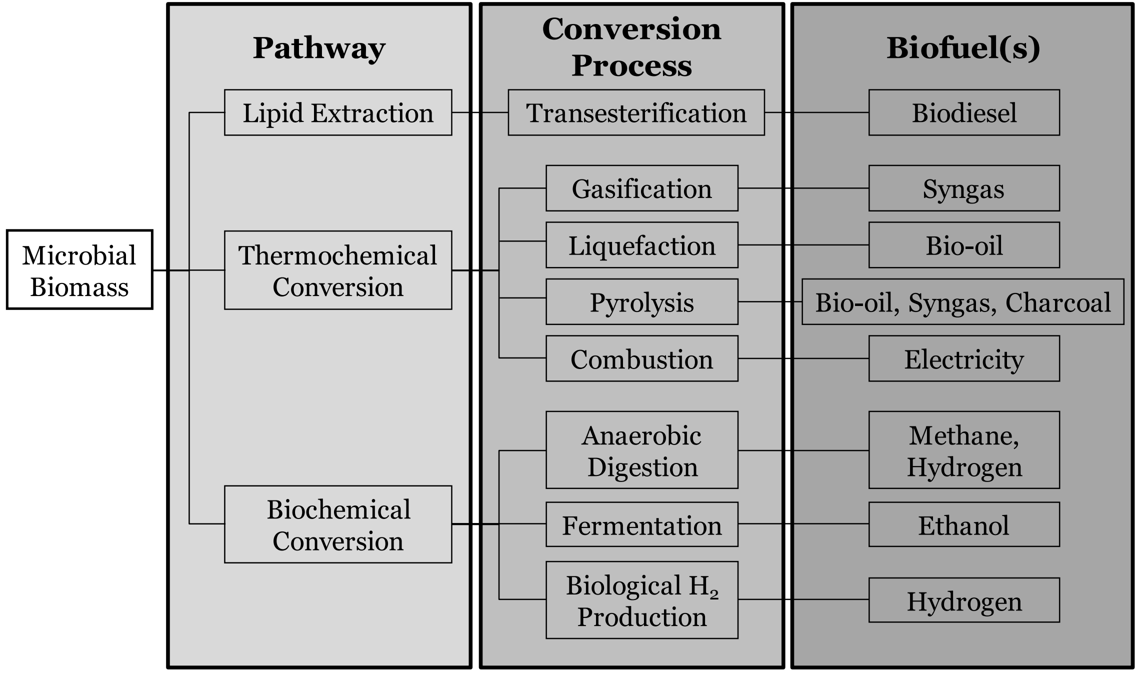

Figure 6-14. Microbial Biomass Conversion Pathways for Biofuels.

(Note: modified from Brennan and Owende [2010].)

Transesterification and anaerobic digestion to produce biodiesel and CH_4, respectively, currently appear to be the most promising channels for microbial biofuels (Huber et al. 2006; Rulkens 2008). Transesterification involves lipid extraction from the biomass (Ma and Hanna 1999; Van Gerpen et al. 2004) while anaerobic digestion can utilise intact biomass (Parkin and Owen 1986). Bio-oil production via thermochemical liquefaction was also addressed below since it is one of the intended outputs of the HRAP demonstration at CWTP (NIWA 2009). Other processes such as combustion, pyrolysis, and gasification require energy-intensive drying that is unlikely to be offset by fuel production in order to be sustainable. Moreover, production of ethanol and hydrogen from sewage sludge has not received much interest, probably due to greater complexities and poorer yields compared to well-established biogas production (Rulkens 2008). Research into these and other methods may expand biofuel options by improving efficiencies and reducing chemical and energy inputs sufficiently (Huber et al. 2006).

Raw wastewater would probably be light limiting for microalgal growth due to high solids content while secondary wastewater was C limiting for bacterial growth. Results presented in this thesis supported the strategy of screening and settling raw wastewater to obtain primary sludge, and then using primary wastewater to grow microbial biomass (or secondary sludge). Therefore, in addition to the yields presented below for the microbial biomass, there is high resource recovery potential from primary sludge. Typically, all sludge is combined at WTPs for biofuel conversion or other uses. However, primary sludge production was not examined during this research, so it has been omitted from the following biofuel estimates.

6.3.1 Biodiesel

Biodiesel can be produced from transesterification of extracted microbial lipids using an alcohol and a catalyst. Approximately 1 kg lipids produces 1 kg biodiesel (Mata et al. 2010). Biodiesel quality is affected by lipid quantity and type (Section 6.2.3). For example, SFAs are generally preferred for biodiesel production (Sheehan et al. 1998), but MUFAs and PUFAs may improve fuel performance depending on location and season since they have better cold temperature properties (Knothe 2005; Sharma et al. 2008). Hence, blending various feedstocks to obtain an optimal lipid profile may offer advantages over the neat form of only one particular type (Knothe 2009). Nonetheless, biodiesel production from this feedstock is not recommended; it would likely be unsustainable due to the low overall lipid content of 7.2-12.6% in solids from primary wastewater cultures. This advice is supported by Sialve et al. (2009), who concluded that energy recovered via lipid extraction from biomass containing < 40% lipids (and even considering biogas production from anaerobic digestion of the residues) is unlikely to compensate for the intensive harvesting, drying, and processing requirements. According to a maximum TSS productivity of 98 $g/m^3/d$, approximately 16,800 kg/d of microbial TSS could be produced at CWTP to generate 2,100 kg/d of lipids based on a wastewater flow of 171,000 m^3/d

(Table 6-5). Lipid generation would reduce to 1,400 kg/d based on a mean settleable TSS productivity of 65 g/m3/d (Table 6-5).

Table 6-5. Estimated Resource Potential of Microbial Biomass Produced from Primary Wastewater at CWTP.

Product	Unit	Potential from 98.1 g/m3/d Total TSS Productivity[a,b]	Potential from 64.8 g/m3/d Settleable TSS Productivity[b]	Assumptions
Biomass	kg/d	16,800	11,100	171,000 m3/d wastewater
Biodiesel	kg/d	2,100	1,400	12.6% lipids in biomass
Biogas / CH_4	m3/d	8,400 / 5,700	5,500 / 3,800	0.5 m3 biogas/kg biomass containing 68% CH_4
Bio-oil	kg/d	10,700	7,100	64% bio-oil conversion

Note: assumed (a) 100% settleability and (b) no loss during harvesting from Table 6-3; additional yields possible from primary sludge conversion.

6.3.2 Biogas

Biogas consisting predominantly of CH_4 and CO_2 can be produced from microbial degradation of biomass in the absence of oxygen. Most biomass is suitable for anaerobic digestion. Generally, a C/N ratio of 20-30 is recommended for optimal CH_4 yields. Microalgae and sewage sludge typically have C/N ratios ranging from 6-16 (Ward et al. 2008). Microbial biomass produced by this research had a C/N ratio of approximately 5. At such low C/N ratios, ammonia and volatile fatty acids (VFAs) can accumulate in digesters, which can decrease methanogenesis (Yen and Brune 2007). However, this limitation can be overcome through co-digestion of higher C/N ratio feedstock (e.g., waste paper products) to improve performance (Yen and Brune 2007; Ward et al. 2008). Ehimen et al. (2011) also successfully increased CH_4 yield (> 50%) of microalgal transesterification residues by co-digesting them with glycerol to increase C/N ratio from 5 to 12. Combined primary and secondary sludge at CWTP has a C/N ratio of about 20, but use of a secondary sludge with higher microalgal content may affect optimal C/N ratios for digestion, so CH_4 production trials are warranted.

Due to the low lipid content of the biomass, it is preferentially suited for biogas production in order to promote sustainability (Sialve et al. 2009). COD conversion of 70-90% from microalgal biomass (Sánchez Hernández and Travieso Córdoba 1993) to produce 0.4-0.6 m3/kg (biogas at normal temperature and pressure) containing 40-68% CH_4 has been demonstrated (Eisenberg 1981; de Schamphelaire and Verstraete 2009). According to a maximum TSS productivity of 98 g/m3/d, 5,700 m3/d of CH_4 could be generated (Table 6-5). Biogas generation would reduce to 3,800 m3/d of CH_4 based on a mean settleable TSS productivity of 65 g/m3/d (Table 6-5).

6.3.3 Bio-oil

Production of bio-oil via thermochemical liquefaction also has potential for net energy gain since wet feedstock can be used. Microalgal bio-oil conversions of 33-64% are possible

(Sawayama et al. 1999; Yang et al. 2004; Demirbas 2010). Hence, according to TSS productivities of 65 to 98 g/m³/d (Table 6-3), bio-oil production in the range of 7,100 to 10,700 kg/d could be expected. Bio-oil can be upgraded via several routes to replace diesel and gasoline as summarised by Huber et al. (2006). Some properties that negatively affect bio-oil quality are low heating value, blending incompatibilities, solids' contents, high viscosity, incomplete volatility, and chemical instability (Huber et al. 2006), so this biomass would need further testing to better gauge its applicability for bio-oil production.

6.4 Other Alternatives

Microbial biomass grown on wastewater can be used for various non-biofuel applications particularly when no chemicals are added for harvesting (Table 6-6). Municipal wastewater treatment was demonstrated by this research, and scope also exists for removal of heavy metals (Munoz and Guieysse 2006). Regarding fertilisers, microbial biomass has an advantage over conventional ones in that it slowly degrades to release N (and other nutrients) over time with only about 3% TN available at application (Mulbry et al. 2005) and 15% released in the first year (Gray 2004). Additionally, applying dried biomass to land avoids ammonia volatilisation and tillage, which can be typical for manure (Mulbry et al. 2005). Since 200 kg N/ha/yr and 20 kg P/ha/yr (as mineral fertiliser) are generally used for intensive agriculture (Haygarth and Jarvis 2002), this would require about 2,400 kg/ha/yr biomass to meet the N requirement while also supplying 40 kg P/ha/yr (i.e., assuming 8.4% TKN and 1.8% TP in biomass; Section 6.2.5). Following biogas production, anaerobic digester residues also have potential for use as fertiliser (Hanisak et al. 1980; Uysal et al. 2010). The residues have a higher N content than undigested biosolids since much of the C is off-gassed during digestion, and up to 70% of organic N is biologically converted to ammonium (NH_4^+), which is readily available to plants (Gray 2004). Land application must consider these nutrient-release characteristics to improve agricultural management strategies.

Nutritionally, protein content and digestibility of biomass are concerns for animal feed. Biomass protein was estimated as 50% from TKN content (Section 6.2.5) based on a conversion factor of 5.95 derived by González López et al. (2010). About 30% of the world's production of microalgae is used as animal feed to supplement or substitute conventional sources (e.g., fishmeal, soybeans, etc.) (Belay et al. 1996). Digestibility is impacted by biomass drying, processing, and storage as well as consumer physiology (i.e., ruminant or non-ruminant). For example, *Scenedesmus* sp. may only be 30% digestible when fresh, but 80% digestible when drum-dried (Becker 1994). In addition, ruminants (e.g., sheep, cattle) are better equipped to digest the cellulosic cell wall of green microalgae, so there is greater possibility to feed untreated biomass directly to these animals compared to non-ruminants (e.g., pigs, horses), which require biomass processing to improve digestibility (Becker 1994).

Table 6-6. Non-Biofuel Applications for Microbial Biomass Grown on Municipal Wastewater.

Application	Examples
Wastewater treatment	Reduction of COD, N, P, heavy metals, and supernatant TSS
Fertilisers	Biofertilisers and soil conditioners for agriculture
Animal feed	Feed supplement for poultry, ruminants, pigs, and fish
Therapeutics	β-carotene as possible skin cancer preventative; linolenic acid to stimulate prostaglandin synthesis
Pigments	β-carotene and phycobilins as food colouring
Fine chemicals	Glycerol use in foods, cosmetics, and pharmaceuticals; fatty acids, lipids, waxes, sterols, hydrocarbons, enzymes, vitamins; polysaccharides as gums
Hormones	Auxins, gibberllins, and cytokines

(Sources: Becker 1994; Richmond 2004)

These and other pathways such as therapeutics, pigments, and hormones have been explored in-depth by others (Cresswell et al. 1989; Becker 1994; Richmond 2004). Biomass use as fertiliser would need to meet site-specific regulations (e.g., pathogens and biosolids [U.S. EPA 1994]), and consumption applications would require nutritional studies (e.g., protein efficiency, digestibility, toxicology, supplementation evaluation) (Becker 1994; Richmond 2004).

6.5 Summary

In summary, the following trends regarding biomass characteristics and bioenergy potential were derived from laboratory-scale microbial cultures fed with municipal wastewater:

- Microbial wastewater cultures were usually dominated by *Scenedesmus* spp. and/or *Micractinium* sp. Climate and wastewater appeared to have some influence on (non-dominant) microbial ecology, but no overriding trends were found relating to biomass productivity, settleability, or composition.
- The microbial community of overloaded SBRs was imbalanced following AS addition. More conservative inoculation of 2% v/v AS achieved greater microalgal diversity, productivity, and microalgal-bacterial symbiosis.
- Solids were 90% organic and contained approximately 45% C, 8% N, and 2% P.
- Biofuel energetics indicated that microbial biomass produced during this research may be best suited for anaerobic digestion (CH_4) or thermochemical liquefaction (bio-oil) due to its relatively low lipid content since these processes use the entire biomass. Accordingly, maximum biofuel estimates for CWTP were 5,700 m^3/d of CH_4 and 10,700 kg/d of bio-oil based on results of this research and current conversion efficiencies.
- Microbial biomass and/or residues from biofuel conversion also have potential for use as animal feed, fertiliser, and other alternatives, which broadens its utilisation and makes it a versatile resource.

Chapter 7: Conclusions

7.1 Research Summary

Microbial (microalgal-bacterial) biomass was cultivated in laboratory reactors for biofuel potential. Experimental designs incorporated sustainability and embraced site-specific conditions of climate, nutrients, and ecology to develop practical, complex microbial communities found in nature. As detailed in previous chapters, productivity, settleability, and composition of the microbial communities were quantified for factors of municipal wastewater, climate, and retention times. New practical knowledge was gained in biomass growth and harvesting, inoculum production, culture stability, wastewater treatment, and engineering designs. New theoretical knowledge was gained relating to biofuel conversion processes and co-products. Synthesised within this chapter, this new knowledge spans seven of the ten research areas necessary for advancing the field of microbial biotechnology as identified by Benemann et al. (2003a).

7.2 Critical Performance Indicators

Biomass productivity, settleability, and composition were identified as the critical variables affecting economic feasibility of large-scale microbial biofuel production processes. Laboratory experiments sequentially maximised productivity under site-specific conditions while enhancing settleability for improved harvesting potential.

7.2.1 Biomass Productivity

Biomass productivity was impacted by climate, C content of municipal wastewater, and retention times. Up to 200% greater biomass was produced in SBRs operated under a warmer climate (i.e., 21 °C mean water temperature with 925 µmol/m^2/s PAR for 14.7 h/d), confirming that microbial growth was limited by lower light and/or temperature experienced under a colder climate (i.e., 13 °C mean water temperature with 410 µmol/m^2/s PAR for 9.6 h/d). Secondary wastewater was a poor substrate for microbial growth most likely because of C limitation, bacterial inhibition at elevated prevailing pH, and reduced photosynthetic efficiency of microalgae at supersaturated DO levels. SBRs inoculated with standardised oxidation pond water and 2% AS and fed with primary wastewater optimised biomass productivity at 21.6 g/m^2/d on average for 2-d HRT and 12-d SRT under warm climatic conditions. Solids washout indicated that 2-d HRT may be near the maximum loading limit of the systems.

Novel assessments of COD utilisation and growth rate modelling for microalgal-bacterial cultures were conducted. Maximum yield up to 0.95 g cell COD/g COD_{used} for 4-d HRT and 4-d SRT cultures fed with primary wastewater and operated under warm climatic conditions indicated almost 50% greater C fixation compared to conventional AS systems. Growth rate modelling supported COD utilisation results by indicating that heterotrophy was mostly C

limited at long (≥ 4-d) HRT and DO limited at short (≤ 2-d) HRT while photoautotrophy was probably always light limited.

7.2.2 Biomass Settleability

Biomass settleability indicated harvestability using sedimentation. It was facilitated by microbial aggregation into stable, compact flocs over time and also by bioflocculation during sedimentation periods. These mechanisms were largely influenced by wastewater loading and microbial growth rate. Poor biomass settleability was observed during exponential growth, but it improved through increased bioflocculation as microbial growth rate decreased. Larger flocs existed in primary wastewater cultures which consistently demonstrated greater settleability compared to secondary wastewater cultures. Settleability was maximised with AS addition and biomass recycling to the range of 68-98%. It was generally greater and more consistent at longer SRT and shorter HRT.

Settleability was affected by light, sedimentation duration, sedimentation container (i.e., size, material, and/or shape), and other factors as examined in complementary studies. Darkness enhanced settleability due to increased bioflocculation, and sedimentation periods could be reduced from 60 to 30 min with comparable performance due to a rapid settling rate. Operational strategies should take these effects into account in order to maximise harvesting efficiency. Additionally, settleability in 1-L, plastic Imhoff Cones was 3% less than in 28-L, steel reactors. Therefore, monitoring methods should be aware of potential differences between estimated and *in-situ* settleabilities in order to reliably indicate harvestable yield.

7.2.3 Settleable Productivity

Offering a novel, simple approach to data presentation, the term 'settleable productivity' was coined to quantify the biomass potentially harvestable following sedimentation and decantation. Settleable productivity was greatest at 20.6 $g/m^2/d$ for 2-d HRT and 12-d SRT under warm climatic conditions, which is within the range reported by others worldwide, but beats reported yields for NZ. Consistent laboratory conditions probably largely contributed to this value, so realistic yields should be verified by outdoor demonstration. Although high productivity and settleability of microbial cultures can be mutually exclusive due to rapid growth at short SRT preventing floc formation, this research demonstrated greatest productivity and settleability concurrently by utilising SBRs to recycle settleable flocs and achieve longer SRT than HRT. It was not necessary to compromise between these two critical parameters to maximise harvestable yield since biomass recycling reduced the overall growth rate of more total biomass at longer SRT and thereby facilitated excellent floc formation and sedimentation at shorter HRT. A novel wastage model was developed to accurately maintain retention times of cultures based on real-time settleability, which strengthened results.

7.2.4 Microbial Composition

Microbial wastewater cultures were usually dominated by *Scenedesmus* spp. and/or *Micractinium* sp. Climate, wastewater, and retention times influenced self-design of microbial communities, but no trends were found relating ecology to biomass productivity or settleability. Solids were 90% organic and contained approximately 45% C, 8% N, and 2% P. Lipid quantities (7.2-12.6%) and profiles did not appear to be substantially impacted by climate or wastewater.

7.3 Bioenergy Implications

For greatest energetic potential, microbial biomass produced by these research methods is probably best suited for entire biomass conversion via anaerobic digestion or thermochemical liquefaction. According to a maximum TSS productivity of 98 $g/m^3/d$, approximately 16,800 kg/d of microbial TSS could be produced from a primary wastewater flow of 171,000 m^3/d at CWTP to generate 5,700 m^3/d of CH_4 or 10,700 kg/d of bio-oil based on current conversion efficiencies. These estimates do not include additional biofuel yields that could be obtained from conversion of primary sludge. Intact biomass and residues from biofuel conversion also have potential for use as animal feed, fertiliser, and other alternatives (although not explored by this research), which make it a versatile and valuable resource.

7.4 Sustainability

7.4.1 Wastewater Treatment

Wastewater was a nutrient-rich medium for microbial growth, and it offered a more sustainable alternative to artificial sources. Microbial cultures were not optimised for wastewater treatment purposes, so water quality discharge levels were not consistently met as this was beyond the scope of this research. However, level of wastewater treatment, settleability, and productivity are related, and they must be managed in large-scale systems to sustainably grow microbial biomass on wastewater for biofuels. Aerobiosis and treatment capacity were sensitive to microbial symbiosis, which was affected by microalgal oxygenation, bacterial respiration, diffusion, and other factors. Cultures containing excess AS inoculum or those operated at short (<4-d) HRT were DO limited, but wastewater degradation was nevertheless supported to varying degrees without supplemental aeration as evident by nutrient removal (e.g., COD, ammonia, TSS) and modelling results. Effluent reuse, secondary nutrient recovery, and/or other alternatives should be considered to consistently meet discharge regulations.

7.4.2 Integrated Systems Approach

High biomass productivity and efficient harvesting are currently recognised challenges in microbial biofuel applications that were addressed using ecological engineering principles and an integrated systems approach (Section 1.5). Combined, symbiotic growth of native microalgae and bacteria promoted efficient O_2/CO_2 exchange to improve productivity and enhanced floc formation to improve settleability compared to purely microalgal or bacterial cultures while

concurrently treating genuine municipal wastewater. In addition to wastewater treatment, other processes could be integrated into full-scale microbial biomass production systems to increase their sustainability. Nutrient recovery could be further maximised with feedback streams as illustrated in Figure 7-1. The current strategy of screening raw wastewater for primary sludge, and then using primary wastewater and secondary nutrient recycling for microbial growth in HRAPs could increase biomass production over conventional AS processes for greater biogas production. Biomass yield up to 0.95 g cell COD/g COD_{used} for these microbial biomass systems indicated almost 50% greater C fixation compared to conventional AS systems (i.e., 0.64 g cell COD/g COD_{used} [Orhon et al. 2009]) even without any feedback streams.

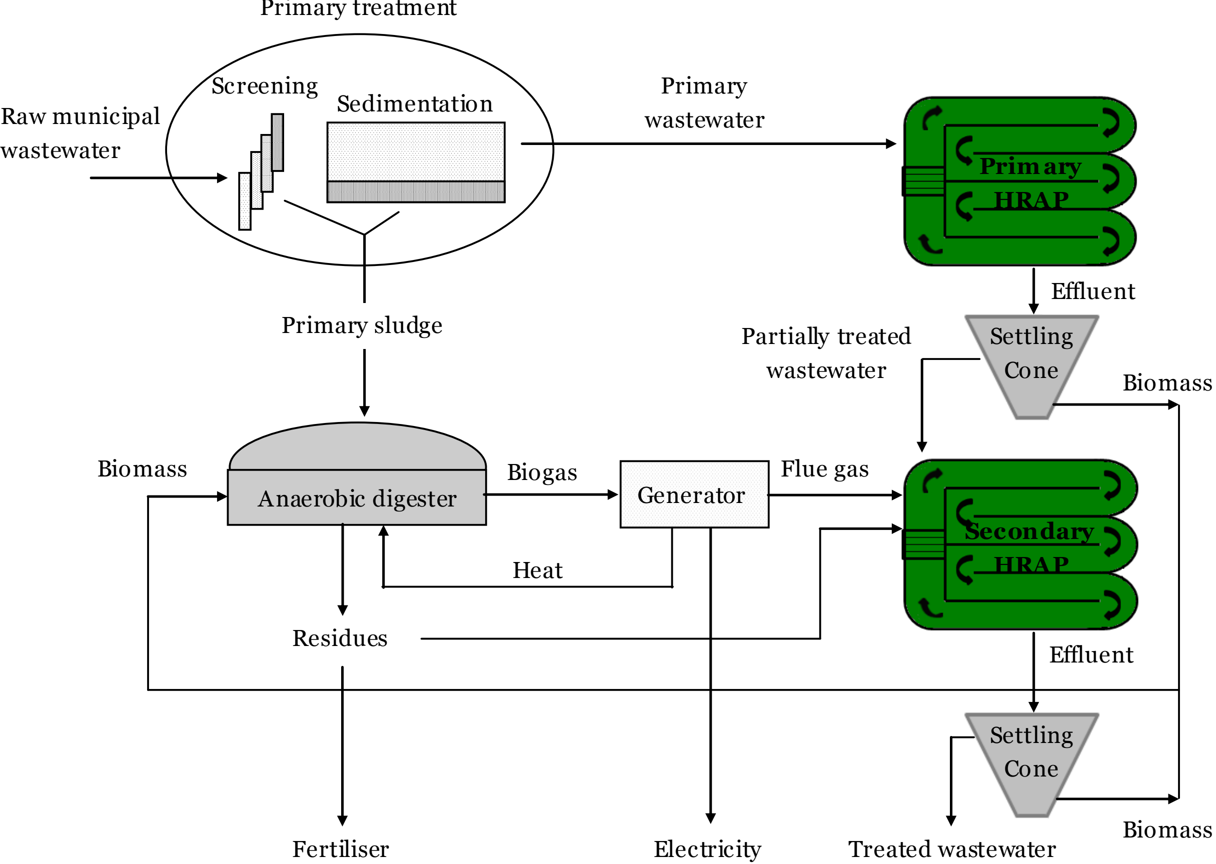

Figure 7-1. Integrated Nutrient Recovery System for Microbial Biomass Production from Municipal Wastewater.

Anaerobic digestion was selected as the conversion process for an integrated microbial biogas production system since it is a proven technology that is already used at some WTPs including CWTP. This process requires a combination of high C/N content biomass (e.g., municipal solid waste, primary sludge) and microbial biomass in order to attain an overall C/N ratio of 20-30 for optimal CH_4 production. Residues and flue gas (containing CO_2) from the digester could be recycled to the HRAP for secondary nutrient recovery (Park and Craggs 2010), and some residues could potentially be used as fertiliser (Hanisak et al. 1980; Uysal et al. 2010).

7.5 Future Research Recommendations

Additional research is recommended to refine microbial biomass technology as summarised in Table 7-1. Although the topics below have been examined for a range of microbial cultures at different locations and various levels of detail in the literature, site-specific studies remain necessary to project performance due to microbial, climate, and feed water variations as demonstrated by this research. Future laboratory research should build on this work by further exploring operational parameters (e.g., SBR cycle duration, culture depth) and feed water characteristics (e.g., COD fractionation) to enhance productivity and wastewater treatment of cultures. Afterwards, biomass conversion studies and outdoor demonstrations are warranted to verify yields. Additionally, integrated systems incorporating secondary nutrient recycling should be investigated to reduce losses (e.g., heat, nutrients) and increase sustainability. Laboratory and outdoor demonstration results should then be evaluated using an LCA or other sustainability estimator (and a cost-benefit analysis) prior to full-scale implementation.

Table 7-1. Future Research Recommendations for Small-Scale Studies Exploring Microbial Biomass Technology.

Topic	Description	Research Question(s)
Diurnal wastewater treatment	Examine how treatment varies during the day and night to determine if multiple SBR cycles per day are beneficial.	Can greater productivity and/or lower HRT be achieved with different cycling strategies?
Loading depth	Examine the effect of culture depth on biomass productivity and oxygenation while maintaining consistent retention times.	Will reduced depth improve light penetration and oxygenation of cultures? What is the recommended loading depth?
COD fractionation	Examine the nature of the COD in the influent and treated wastewaters.	Can soluble COD utilisation be improved to < 50 mg/L to meet discharge requirements?
Biomass conversion processes	Verify bio-oil and biogas yields from biomass conversion and determine blending requirements to optimise CH_4 production.	How do these yields compare with those of activated sludge and other feedstocks?
Secondary nutrient recycling	Examine critical performance indicators of reactors fed anaerobic digester effluent, secondary wastewater, and CO_2 (i.e., flue gas).	Will secondary nutrient recycling improve wastewater treatment? What feeding ratios/strategies promote highest productivity?
Outdoor demonstration	Apply sequentially optimised operational conditions of laboratory reactors to pilot-scale HRAP(s) and monitor performance.	How do results compare for laboratory and outdoor studies? Are operational adjustments required?
Life cycle assessment	Estimate input and output energies associated with microbial biofuel production and/or other products (e.g., fertilisers).	How do biogas and bio-oil sourced from microbial biomass compare with other (bio)fuel feedstocks?

Finding sustainable solutions to limited planetary resources such as energy, water, and land will benefit humanity's quality of life and health. There will not be one all-encompassing solution to meet energy challenges across the globe, but rather a myriad of renewable energy applications depending on site-specific characteristics coupled with reduced energy usage overall from more sustainable lifestyles. Microbial biomass production from municipal wastewater is a promising technology that can be refined with further research to aid this strategy by substantially contributing to bioenergy supplies.

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Appendix A. Microbial Identification

A.1 Light Microscopy of Microalgae and Microfauna

Figure A-1. Photomicrographs of Microalgae and Microfauna in Cultures.

Common Name: Phylum, Order, Species	Photomicrograph(s)
Green microalgae: Chlorophyta, Chlorococcales, Coelastrum sp.	
Green microalgae: Chlorophyta, Chlorococcales, Dictyosphaerium cf. pulchellum	
Green microalgae: Chlorophyta, Chlorococcales, Micractinium sp.	
Green microalgae: Chlorophyta, Chlorococcales, Pediastrum sp.	
Green microalgae: Chlorophyta, Chlorococcales, Scenedesmus sp.	
Green microalgae: Chlorophyta, Chlorococcales, cf. Schroederia sp.	
Green microalgae: Chlorophyta, Chlorococcales, cf. Selenastrum sp.	
Green microalgae: Chlorophyta, Chlorococcales, cf. Monoraphidium sp. or Kirchneriella sp.	
Green microalgae: Chlorophyta, Chlorococcales, cf. Monoraphidium sp.	
Green microalgae: Chlorophyta, Chlorococcales, Pseudococcomyxa simplex	
Green microalgae: Chlorophyta, Volvocales, cf. Eudorina sp.	
Green microalgae: Chlorophyta, Volvocales, cf. Chlamydomonas sp.	

Common Name: Phylum, Order, Species	Photomicrograph(s)
Cyanobacteria: Cyanophyta, Unknown	
Euglenoid: Euglenophyta, Euglenales, *Lepocinclis cf. texta*	
Diatom: Bacillariophyta, Bacillariales, *Nitzschia* sp.	
Protozoa: Ciliophora, Sessilida, *Vorticella* sp.	
Animalia: Rotifera, Unknown	
Roundworm: Nematoda, Unknown	

Table A-1. Presence and Dominance of Microalgae and Microfauna Examined Using Light Microscopy. (Note: X = present; D = dominant; 1 (>) 2 (>) 3 = dominance level.)

Common Name: Phylum, Order	**Source**	P1	P6	C1	C6F	C6	P6	SBR1
	Sampling Date	24/01/08	24/01/08	29/01/08	29/01/08	29/01/08	07/02/08	18/02/08
	Species \ Section	3.1	3.1	3.1	3.1	3.1	3.3	3.3
Green algae: Chlorophyta, Chlorococcales	cf. *Ankistrodesmus* sp.							
Green algae: Chlorophyta, Chlorococcales	cf. *Chlorococcum* sp.					X		
Green algae: Chlorophyta, Chlorococcales	cf. *Chlorella* sp., *Choricystis* sp., or *Micractinium* sp.			X	X	X	X	X
Green algae: Chlorophyta, Chlorococcales	*Coelastrum* sp.		X					
Green algae: Chlorophyta, Chlorococcales	*Dictyosphaerium* cf. *pulchellum*			X				
Green algae: Chlorophyta, Chlorococcales	*Micractinium* sp.			X			D	D
Green algae: Chlorophyta, Chlorococcales	*Oocystis* sp.		X					
Green algae: Chlorophyta, Chlorococcales	*Pediastrum* sp.		X				D	D
Green algae: Chlorophyta, Chlorococcales	*Scenedesmus* sp.			X	X	X		D
Green algae: Chlorophyta, Chlorococcales	cf. *Schroederia* sp.							X
Green algae: Chlorophyta, Chlorococcales	cf. *Selenastrum* sp.							
Green algae: Chlorophyta, Chlorococcales	cf. *Monoraphidium* sp. or *Kirchneriella* sp.							
Green algae: Chlorophyta, Chlorococcales	cf. *Monoraphidium* sp.							
Green algae: Chlorophyta, Chlorococcales	*Pseudococcomyxa simplex*							
Green algae: Chlorophyta, Sphaeropleales	cf. *Scotiellopsis*							
Green algae: Chlorophyta, Volvocales	cf. *Eudorina* sp.		X	X	X	X		
Green algae: Chlorophyta, Volvocales	cf. *Chlamydomonas* (or zoospores)							
Green algae: Chlorophyta, Zygnematales	*Closterium* cf. *aciculare*				X			
Green algae: Chlorophyta, Unknown	Unknown green microalgae		X					
Cyanobacteria: Cyanophyta, Unknown	Unknown cyanobacteria							
Euglenoid: Euglenophyta, Euglenales	*Lepocinclis* cf. *texta*	X	X				D	X
Euglenoid: Euglenophyta, Unknown	Unknown euglenoids	X	X			X	X	X
Diatom: Bacillariophyta, Bacillariales	*Nitzschia* sp.			X	X			
Diatom: Bacillariophyta, Unknown	Unknown diatoms							
Protozoa: Ciliophora, Sessilida	Unknown ciliates							
Protozoa: Ciliophora, Sessilida	Vorticella spp.							
Animalia: Rotifera, Unknown	Unknown rotifers							
Roundworm: Nematoda, Unknown	Unknown nematodes							

Table A-1. Presence and Dominance of Microalgae and Microfauna Examined Using Light Microscopy. (Note: X = present; D = dominant; 1 (>) 2 (>) 3 = dominance level.)

Source	SBR2	SBR1	SBR2	P6	R1	R2	R1	R2	P6 inoc
Sampling Date	18/02/08	18/03/08	18/03/08	18/04/08	07/05/08	07/05/08	27/06/08	27/06/08	25/07/08
Species \ Section	3.3	3.3	3.3	3.2	3.2	3.2	3.2	3.2	4
cf. *Ankistrodesmus* sp.					X	X	X	X	
cf. *Chlorococcum* sp.									
cf. *Chlorella* sp., *Choricystis* sp., or *Micractinium* sp.	X	X	X	X	X	X	X	X	X
Coelastrum sp.									
Dictyosphaerium cf. *pulchellum*							X	X	X
Micractinium sp.	D			X			X	X	
Oocystis sp.					X	X			
Pediastrum sp.	D	D	D						
Scenedesmus sp.	D				X	X	D	D	D
cf. *Schroederia* sp.	X						X	X	
cf. *Selenastrum* sp.					X	X	D	D	
cf. *Monoraphidium* sp. or *Kirchneriella* sp.					X	X	X	X	X
cf. *Monoraphidium* sp.									X
Pseudococcomyxa simplex									
cf. *Scotiellopsis*									
cf. *Eudorina* sp.									
cf. *Chlamydomonas* (or zoospores)									
Closterium cf. *aciculare*									
Unknown green microalgae					X	X	X	X	X
Unknown cyanobacteria					X	X			
Lepocinclis cf. *texta*	X								X
Unknown euglenoids	X			X					X
Nitzschia sp.									
Unknown diatoms		D	D				X	X	
Unknown ciliates									
Vorticella spp.									
Unknown rotifers									
Unknown nematodes									

Table A-1. Presence and Dominance of Microalgae and Microfauna Examined Using Light Microscopy. (Note: X = present; D = dominant; 1 (>) 2 (>) 3 = dominance level.)

Source	T1-CO	T1-SE	T1-PE	T1-AP	P6 inoc	T2-CO	T2-SE	T2-PE	T2-AP
Sampling Date	26/08/08	26/08/08	26/08/08	26/08/08	01/09/08	01/10/08	01/10/08	01/10/08	01/10/08
Species \ Section	4	4	4	4	4	4	4	4	4
cf. *Ankistrodesmus* sp.									
cf. *Chlorococcum* sp.									
cf. *Chlorella* sp., *Choricystis* sp., or *Micractinium* sp.	X	X	X	X	X	X	X	X	X
Coelastrum sp.									
Dictyosphaerium cf. *pulchellum*		X							
Micractinium sp.		X	D	D	D		D	D	D
Oocystis sp.									
Pediastrum sp.				X					
Scenedesmus sp.	D	D	X	D	X		D	D	X
cf. *Schroederia* sp.									
cf. *Selenastrum* sp.			X						
cf. *Monoraphidium* sp. or *Kirchneriella* sp.		X			X			X	
cf. *Monoraphidium* sp.	X	X	X		X				X
Pseudococcomyxa simplex		X	D	X	X			X	
cf. *Scotiellopsis*									
cf. *Eudorina* sp.									
cf. *Chlamydomonas* (or zoospores)									
Closterium cf. *aciculare*									
Unknown green microalgae	X			X					
Unknown cyanobacteria									
Lepocinclis cf. *texta*				X					
Unknown euglenoids						X		X	X
Nitzschia sp.									
Unknown diatoms	X		X	X	X		X	X	X
Unknown ciliates									
Vorticella spp.									
Unknown rotifers									
Unknown nematodes									

Table A-1. Presence and Dominance of Microalgae and Microfauna Examined Using Light Microscopy. (Note: X = present; D = dominant; 1 (>) 2 (>) 3 = dominance level.)

Source	P6 inoc	T3-CO	T3-SE	T3-PE	T3-AP	P6 inoc	T4-CO	T4-SE	T4-PE
Sampling Date	14/10/08	13/11/08	13/11/08	13/11/08	13/11/08	19/01/09	19/02/09	19/02/09	19/02/09
Species \ Section	4	4	4	4	4	4	4	4	4
cf. *Ankistrodesmus* sp.									
cf. *Chlorococcum* sp.									
cf. *Chlorella* sp., *Choricystis* sp., or *Micractinium* sp.	X	X			X				
Coelastrum sp.									
Dictyosphaerium cf. *pulchellum*			X	D	D		X		
Micractinium sp.	X		D	X		D	X	X	X
Oocystis sp.									
Pediastrum sp.						X	X	X	D
Scenedesmus sp.		X		D	X	X	X	D	D
cf. *Schroederia* sp.									
cf. *Selenastrum* sp.									
cf. *Monoraphidium* sp. or *Kirchneriella* sp.	X								
cf. *Monoraphidium* sp.	X								
Pseudococcomyxa simplex	X								
cf. *Scotiellopsis*									
cf. *Eudorina* sp.									
cf. *Chlamydomonas* (or zoospores)	D								
Closterium cf. *aciculare*									
Unknown green microalgae	X					X	X	X	X
Unknown cyanobacteria							X		
Lepocinclis cf. *texta*	X								
Unknown euglenoids									
Nitzschia sp.									
Unknown diatoms			X	X	X				
Unknown ciliates									
Vorticella spp.				X	X				X
Unknown rotifers				X	X				X
Unknown nematodes									X

Table A-1. Presence and Dominance of Microalgae and Microfauna Examined Using Light Microscopy. (Note: X = present; D = dominant; 1 (>) 2 (>) 3 = dominance level.)

Source	T4-AP	P6 inoc	T5-CO	T5-SE	T5-PE	T5-AP	P6 inoc	T6-CO	T6-SE
Sampling Date	19/02/09	26/02/09	27/03/09	27/03/09	27/03/09	27/03/09	02/04/09	01/05/09	01/05/09
Species \ Section	4	4	4	4	4	4	4	4	4
cf. *Ankistrodesmus* sp.									
cf. *Chlorococcum* sp.									
cf. *Chlorella* sp., *Choricystis* sp., or *Micractinium* sp.									
Coelastrum sp.					X				X
Dictyosphaerium cf. *pulchellum*							X		
Micractinium sp.	X	D		D	X	D	X		X
Oocystis sp.									
Pediastrum sp.	D	X		X	X	X	X	X	X
Scenedesmus sp.	D	X	X	D	D	X	D	X	D
cf. *Schroederia* sp.									
cf. *Selenastrum* sp.							X		
cf. *Monoraphidium* sp. or *Kirchneriella* sp.									
cf. *Monoraphidium* sp.									
Pseudococcomyxa simplex									
cf. *Scotiellopsis*			X						
cf. *Eudorina* sp.									
cf. *Chlamydomonas* (or zoospores)									
Closterium cf. *aciculare*									
Unknown green microalgae	X	X	D	X					
Unknown cyanobacteria			X	X				D	
Lepocinclis cf. *texta*									
Unknown euglenoids		X							
Nitzschia sp.				X	X	X			
Unknown diatoms									X
Unknown ciliates				X	X	X			
Vorticella spp.	X			X	X	X			
Unknown rotifers	X			X	X	X		X	X
Unknown nematodes					X				

Table A-1. Presence and Dominance of Microalgae and Microfauna Examined Using Light Microscopy. (Note: X = present; D = dominant; 1 (>) 2 (>) 3 = dominance level.)

Source	T6-PE	T6-AP	P6 inoc	T1-S12	T1-S8	T1-S4	T1-S12	T1-S8	T1-S4
Sampling Date	01/05/09	01/05/09	01/10/09	11/11/09	11/11/09	11/11/09	07/12/09	07/12/09	07/12/09
Species \ Section	4	4	5	5	5	5	5	5	5
cf. *Ankistrodesmus* sp.									
cf. *Chlorococcum* sp.									
cf. *Chlorella* sp., *Choricystis* sp., or *Micractinium* sp.									
Coelastrum sp.									
Dictyosphaerium cf. *pulchellum*		X							
Micractinium sp.			D	3	2	2		X	1
Oocystis sp.									
Pediastrum sp.	X	X					X		
Scenedesmus sp.	D	D	D	1	2	1	1	3	3
cf. *Schroederia* sp.									
cf. *Selenastrum* sp.									
cf. *Monoraphidium* sp. or *Kirchneriella* sp.									
cf. *Monoraphidium* sp.					X				
Pseudococcomyxa simplex									
cf. *Scotiellopsis*									
cf. *Eudorina* sp.									
cf. *Chlamydomonas* (or zoospores)									
Closterium cf. *aciculare*									
Unknown green microalgae									
Unknown cyanobacteria				X	X		3	1	
Lepocinclis cf. texta									
Unknown euglenoids									X
Nitzschia sp.									
Unknown diatoms				2	1	2	2	2	3
Unknown ciliates	X	X			X	X			
Vorticella spp.		X		X	X	X			X
Unknown rotifers	X	X		X	X	X	X		
Unknown nematodes		X				X			

Table A-1. Presence and Dominance of Microalgae and Microfauna Examined Using Light Microscopy. (Note: X = present; D = dominant; 1 (>) 2 (>) 3 = dominance level.)

Source	P6 inoc	T2-S12	T2-S8	T2-S4	T2-S12
Sampling Date	16/12/09	01/03/10	01/03/10	01/03/10	08/04/10
Species \ Section	5	5	5	5	5
cf. *Ankistrodesmus* sp.					
cf. *Chlorococcum* sp.					
cf. *Chlorella* sp., *Choricystis* sp., or *Micractinium* sp.	X	X	2	X	X
Coelastrum sp.		X		X	
Dictyosphaerium cf. *pulchellum*					
Micractinium sp.	1	X	X	X	X
Oocystis sp.					
Pediastrum sp.	X				
Scenedesmus sp.	2	1	1	1	1
cf. *Schroederia* sp.					
cf. *Selenastrum* sp.					
cf. *Monoraphidium* sp. or *Kirchneriella* sp.					
cf. *Monoraphidium* sp.					
Pseudococcomyxa simplex					
cf. *Scotiellopsis*					
cf. *Eudorina* sp.					
cf. *Chlamydomonas* (or zoospores)					
Closterium cf. *aciculare*					
Unknown green microalgae					
Unknown cyanobacteria		X	X	X	X
Lepocinclis cf. texta	X				
Unknown euglenoids					
Nitzschia sp.					
Unknown diatoms		X	3	X	X
Unknown ciliates					
Vorticella spp.					
Unknown rotifers					
Unknown nematodes					

A.2 Bacterial Gene Sequencing Analysis

16S rRNA gene sequencing analysis was conducted by Dr. Susan Turner and Kristi Biswas, collaborators at the Microbial Ecology and Genomics Laboratory, School of Biological Sciences, University of Auckland, NZ. (Note: sample suffix '-B' and '-S' denote biomass and supernatant clone libraries, respectively.)

Table A-2. Bacterial Taxonomy and Clone Library Abundances of Supernatant and Settled Biomass Samples.

Sample	SBR1-B	SBR1-S	AP-B	AP-S	PE-B	PE-S	SE-B	SE-S	CO-B	CO-S	SE-B	PE-B	AP-B
Date	Mar-08	Mar-08	Dec-08	Dec-08	Dec-08	Dec-08	Dec-08	Dec-08	Dec-08	Dec-08	Apr-09	Apr-09	Apr-09
domain Bacteria (xx) (sequences)	**88**	**82**	**95**	**89**	**90**	**90**	**91**	**85**	**94**	**91**	**86**	**88**	**91**
unclassified_Bacteria	4	1	1	3	2			8	1	8	2	1	1
phylum Cyanobacteria		**1**	**1**		**1**	**1**		**8**	**2**	**17**	**22**	**30**	**11**
class cyanobacteria			1		1	1		8	2	17	22	30	11
phylum Proteobacteria	**34**	**56**	**65**	**63**	**62**	**80**	**63**	**38**	**47**	**54**	**47**	**44**	**61**
unclassified_Proteobacteria			2	1		1			1				1
class Alphaproteobacteria	7	1	1	5	3		3	1	11	40	22	14	17
unclassified_Alphaproteobacteria	2			4	1		1	1	2		2		2
order Rhodospirillales										1	2	2	
order Rhodobacterales	2	1			1				2	1	14	7	9
order Rhizobiales	2						2				1	4	4
order Sphingomonadales					1				7	36	2	1	2
order Caulobacterales	1		1							1	1		
order Rickettsiales				1						1			
class Betaproteobacteria	9	49	47	43	47	75	20	32	20	11	9	13	37
unclassified_Betaproteobacteria	9	38	2	2	3	9	3	6	2	2			
order Burkholderiales		2	34	35	43	65	16	22	12	5	9	12	27
Genus Acidovorax		1	3	5	1	3			1				
order Rhodocyclales		9	5	2	1			1	1			1	8
order Nitrosomonadales			1	1			1	2					
order Neisseriales						1		1					1
order Methylophilales			5	3					5	4			
class Gammaproteobacteria	18	6	11	7	8	3	40	4	15	3	16	17	5
unclassified_Gammaproteobacteria			3	2	1	1	1		1			1	
order Xanthomonadales	1	1	2	2	3				1		1		2
order Aeromonadales			1								2	2	1
order Pseudomonadales	1	3	1	2	3	2	2	2	11	2	1	1	
order Legionellales				1					2	1			
order Chromatiales							3						
order Enterobacteriales					1						12	13	2
order Oceanospirillales			1					2					
order Alteromonadales			3				34						
order Thiotrochales	16	2											
class Deltaproteobacteria			1	7	2			1					1
unclassified_Deltaproteobacteria													
order Desulfobacterales				1									1
order Myxococcales				4									
order Bdellovibrionales			1	2	2			1					
class Epsilonproteobacteria			3		2	1							
order Campylobacteraceae			3										
phylum Firmicutes	**40**	**21**					**1**				**5**	**3**	**9**
unclassified_Firmicutes	4												
class "Clostridia"	32	20					1				2	2	9
order clostridiales		19					1				1	2	6
class Mollicutes													
order Incertae sedis8													
class Bacilli	4	1									3	1	
order Bacillales	2	1											
order Lactobacillales	2										3	1	
phylum Actinobacteria	**4**					**3**	**4**	**1**					**3**
unclassified_Actinobacteria													
class Actinobacteria	4					3	4	1					
subclass Actinobacteridae	3					3	4	1					
subclass Coriobacteridae	1												
phylum Planctomycetes	**2**												
unclassified Planctomycetes													
class Planctomycetacia	2												
order Planctomycetales	2												
phylum Bacteroidetes	**3**	**3**	**26**	**21**	**24**	**4**	**21**	**30**	**42**	**11**	**1**	**5**	**4**
unclassified_Bacteroidetes		2	12	15	3		1						
class Bacteroidetes	1				2								
order Bacteroidales	1				2								
class Flavobacteria	1		4		10	2	13	2	8	1		4	2
order Flavobacteriales	1		4		10	2	13	2	8	1		4	2
class Sphingobacteria	1	1	10	6	9	2	7	28	34	10	1	1	2
order Sphingobacteriales	1		10	6	9	2	7	28	34	10	1	1	2
phylum Fusobacteria					**1**	**1**							
class Fusobacteria					1	1							
order Fusobacteriales					1	1							
phylum Verrucomicrobia	**1**		**2**	**2**		**1**	**1**						
class Verrucomicrobiae	1		2	2		1	1						
order Verrucomicrobiales	1		2	2		1	1						
phylum OP10											**9**	**4**	
Others							1		2	1		1	3

Appendix B. Supplementary Materials and Methods

B.1 Feed Water Degradation

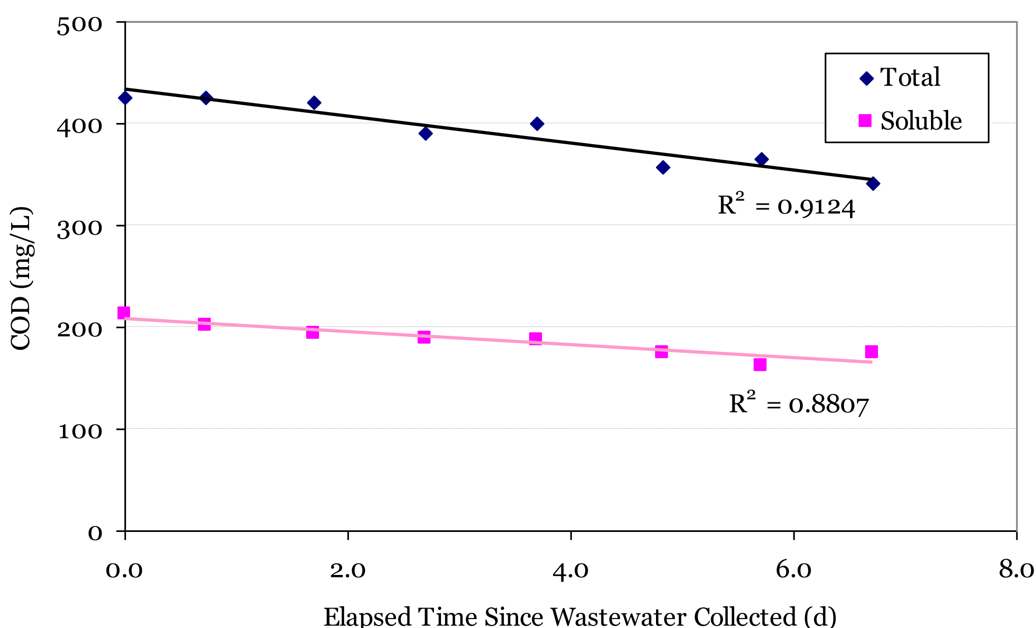

Figure B-1. Degradation of COD in Primary Wastewater over Time.

B.2 Mixing Efficiency of Reactors

Table B-1. Examination of TSS of Mixed Cultures Collected Near Top and Bottom of Water Column in Steel Reactors.

Parameter / Trial No.	1	2	3	4	5	6	7	8	9
Reactor Top TSS (mg/L)	210	172	222	433	389	168	153	246	467
Reactor Bottom TSS (mg/L)	194	184	222	432	372	181	151	245	424
Standard Deviation (mg/L)	11	8	0	1	12	9	1	1	30
RPD (%)	7.9	6.7	0.0	0.2	4.5	7.4	1.3	0.4	9.7

B.3 SBR Operating Sequence Details

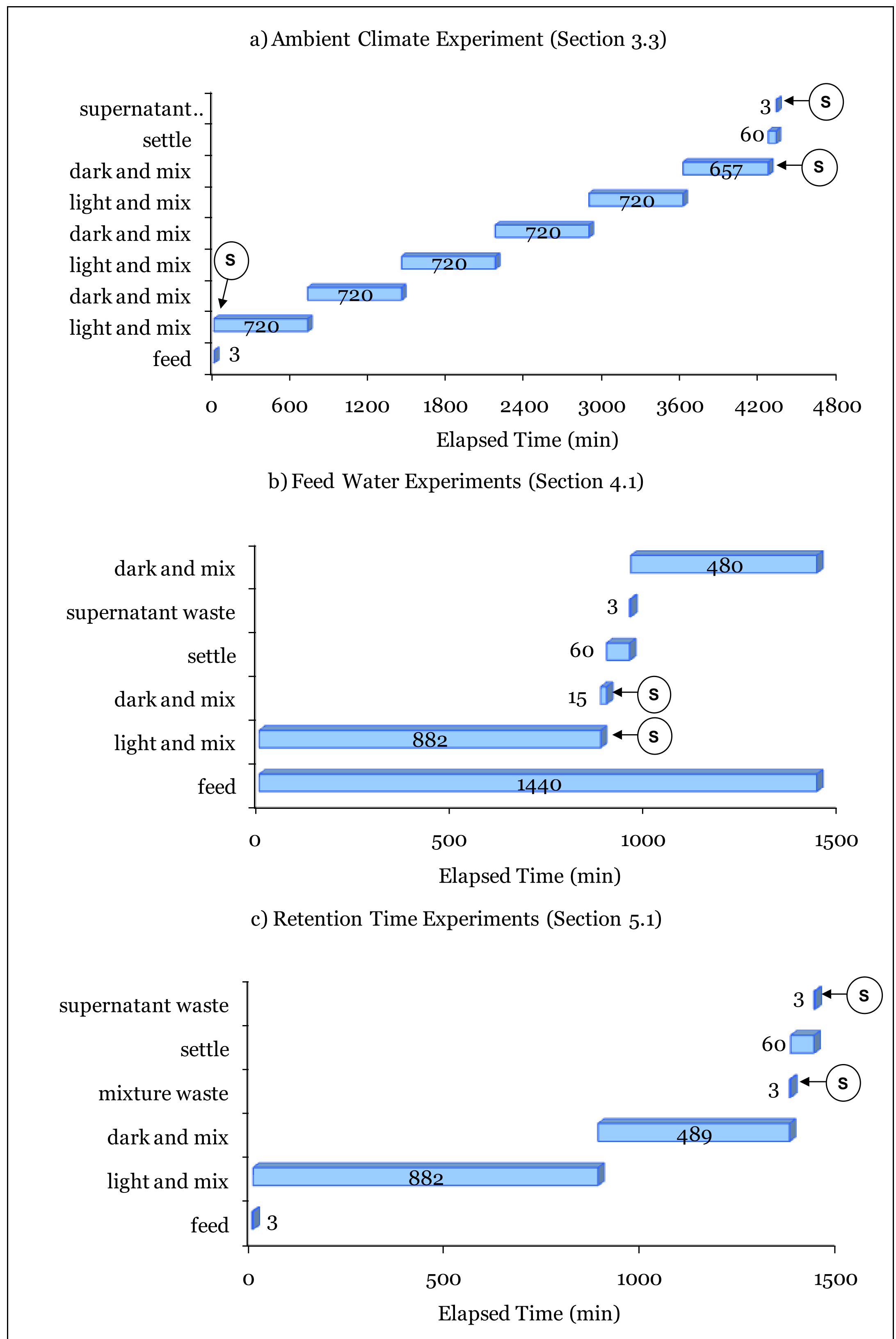

Figure B-2. Regular Operating Sequences and Step Durations of SBR Experiments.

(Note: S = sample collected for analyses.)

B.4 COD Analysis

This laboratory method was modified from SM 5220D (APHA 2005) .

B.4.1 Solution Preparation

- COD Standard (1,200mg/L): Dissolve 1.0213 g KHP (dried at 100 °C for 1 h) in deionised water and dilute to 1 L.
- Sulphuric Acid Reagent: Add 25.3 g Ag_2SO_4 to 2.5 L H_2SO_4. Stand for 48 h to thoroughly dissolve.
- Digestion Solution A: Combine 10.216 g $K_2Cr_2O_7$, 167 mL H_2SO_4, and 33.3 g $HgSO_4$ in 500 mL deionised water and dilute to 1 L.
- High Range Digestion Solution: Add 150 mL Solution A to 350 mL Sulphuric Acid Reagent in a water bath with care.

B.4.2 Method Development

The mean absorbance values (Table B-2) were used to create a calibration curve (Figure B-3) and a customised programme (#951) in the Hach DR/2000 spectrophotometer. The accuracy of the low end of the curve was also verified down to 200 mg/L using a 200 mg/L KHP QC standard (i.e., 4% error). Low range COD (< 150 mg/L) analyses were conducted in accordance with Hach (2003) using standard reagents.

Table B-2. COD Standards, Absorbances, and Results.

COD Standard (mg/L)	Absorbance 1	Absorbance 2	Mean Absorbance	Post-Digestion COD (mg/L)
300	0.111	0.084	0.0975	308
600	0.192	0.176	0.184	610
900	0.286	0.286	0.286	912
1,200	0.400	0.356	0.378	1,199

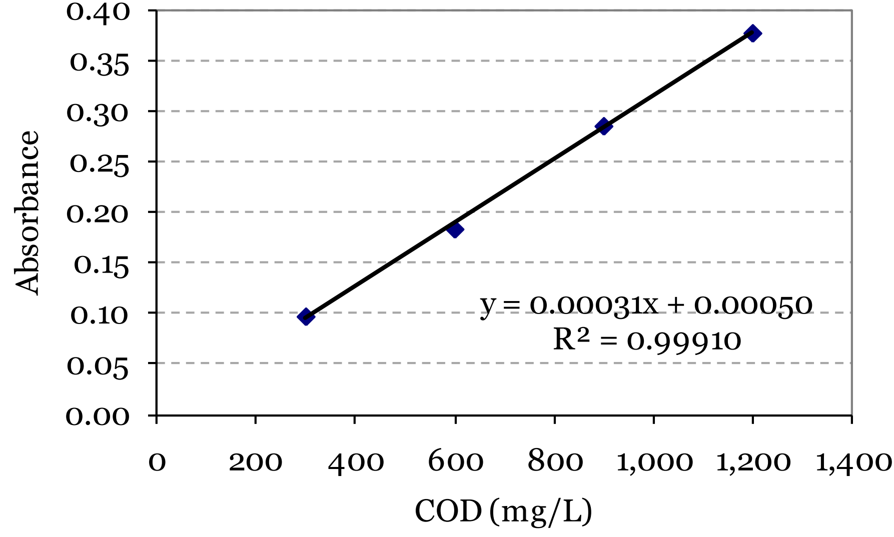

Figure B-3. COD Calibration Curve.

B.5 QA/QC Data

Table B-3. QA/QC Data of Samples, Standards, and Blanks.

Analyte (Range [mg/L])	RPD (%)	Error (%)	Sampling Date
Chl *a*	6		3/04/08
Chl *a*	9		3/04/08
Chl *a*	9		3/04/08
Chl *a*	18		3/04/08
Chl *a*	6		3/04/08
Chl *a*	0		3/04/08
Chl *a*	24		3/04/08
Chl *a*	6		3/04/08
Chl *a*	3		3/04/08
Chl *a*	11		21/08/08
Chl *a*	2		30/10/08
Chl *a*	9		6/11/08
Chl *a*	7		16/02/09
Chl *a*	12		26/03/09
Chl *a*	39		26/03/09
Chl *a*	18		26/03/09
Chl *a*	5		26/03/09
Chl *a*	13		30/04/09
Chl *a*	8		30/04/09
Chl *a*	1		30/04/09
Chl *a*	16		30/04/09
Chl *a*	17		30/04/09
Chl *a*	2		30/04/09
Chl *a*	6		30/04/09
Chl *a*	15		30/04/09
Chl *a*	0		30/04/09
Chl *a*	13		30/04/09
Chl *a*	41		1/10/09
Chl *a*	8		1/10/09
Chl *a*	18		20/10/09
Chl *a*	40		29/10/09
Chl *a*	8		2/11/09
Chl *a*	41		12/11/09
Chl *a*	26		16/11/09
Chl *a*	55		23/11/09
Chl *a*	3		30/11/09
Chl *a*	11		7/12/09
Chl *a*	5		4/01/10
Chl *a*	13		6/01/10
Chl *a*	11		14/01/10
Chl *a*	7		28/01/10
Chl *a*	12		18/02/10
Chl *a*	1		25/02/10
Chl *a*	4		2/03/10
Chl *a*	0		29/03/10
COD (0-200)	2		5/02/08
COD (0-200)	3		29/02/08
COD (0-200)	11		25/08/08
COD (0-200)	14		29/09/08
COD (0-200)	2		17/11/08
COD (0-200)	5		17/11/08
COD (0-200)	21		30/03/09
COD (0-200)	8		4/05/09
COD (0-200)	5		20/10/09
COD (0-200)	2		5/11/09
COD (0-200)	27		9/11/09

Analyte	RPD (%)	Error (%)	Sampling Date
COD (0-200)	0		23/11/09
COD (0-200)	2		26/11/09
COD (0-200)	5		3/12/09
COD (0-200)	5		6/01/10
COD (0-200)		8	8/02/10
COD (0-200)	12		22/02/10
COD (0-200)	3		22/02/10
COD (0-200)	5		8/03/10
COD (0-200)	1		8/03/10
COD (0-200)	16		26/03/10
COD (200-1200)	6		29/02/08
COD (200-1200)	3		15/03/08
COD (200-1200)	3		30/03/09
COD (200-1200)	3		4/05/09
COD (200-1200)	3		15/10/09
COD (200-1200)	2		29/10/09
COD (200-1200)	0		12/11/09
COD (200-1200)	7		26/11/09
COD (200-1200)	3		3/12/09
COD (200-1200)	4	0	12/01/10
COD (200-1200)	1	2	12/01/10
COD (200-1200)	2		21/01/10
COD (200-1200)	14		8/02/10
COD (200-1200)	18		16/02/10
COD (200-1200)	1		25/02/10
COD (200-1200)	0	-3	26/02/10
COD (200-1200)	0	10	26/02/10
COD (200-1200)	2		11/03/10
COD (200-1200)	4		26/03/10
Mass	0		16/03/09
Mass	0		23/03/09
Mass	1		30/03/09
Mass	1		27/04/09
Mass	1		30/04/09
NH_3	6		25/08/08
NH_3 (<1)	67		29/09/08
NH_3 (<1)	86		4/05/09
TC (100-4000)		2	2/10/09
TC (100-4000)		-6	7/10/09
TC (100-4000)		-8	15/10/09
TC (100-4000)		-12	4/03/10
TC (10-500)		16	6/10/09
TC (10-500)		-2	15/10/09
TC (10-500)		31	22/10/09
TC (10-500)		-8	5/11/09
TC (10-500)		5	26/11/09
TC (10-500)		-9	21/01/10
TC (10-500)		-1	4/02/10
TC (10-500)		-10	18/02/10
TC (10-500)		-8	25/02/10
TC (5-200)		-5	27/10/09
TC (5-200)		-4	27/10/09
TC (5-200)		-3	5/11/09
TC (5-200)		-1	19/11/09
TC (5-200)		-3	6/01/10
TC (5-200)		-3	21/01/10

Shading indicates value outside QA/QC limit.

Table B-3. QA/QC Data of Samples, Standards, and Blanks.

Analyte (Range [mg/L])	RPD (%)	Error (%)	Sampling Date
TOC (100-4000)		-5	9/10/09
TOC (100-4000)		13	15/12/09
TOC (10-500)		-1	1/10/09
TOC (10-500)		-1	20/10/09
TOC (10-500)		-4	29/10/09
TOC (10-500)		13	6/11/09
TOC (10-500)		11	26/11/09
TOC (10-500)		7	6/01/10
TOC (10-500)		30	18/02/10
TOC (10-500)		4	25/02/10
TOC (1-50)		-12	28/07/09
TOC (1-50)		-14	6/10/09
TOC (1-50)		15	20/10/09
TOC (1-50)		12	29/10/09
TOC (1-50)		15	6/11/09
TOC (1-50)		1	19/11/09
TS	2		27/03/08
TS	0		17/04/08
TS	13		25/08/08
TS	4		25/08/08
TS	9		25/08/08
TS	1		25/08/08
TS	13		20/02/09
TS	6		20/02/09
TS	5		4/05/09
TS	0		9/04/10
TSS	1		17/02/08
TSS	14		26/02/08
TSS	2		26/02/08
TSS	5		29/02/08
TSS	0		29/02/08
TSS	3		3/03/08
TSS	1		12/03/08
TSS	0		3/04/08
TSS	0		3/04/08
TSS	23		15/04/08
TSS	41		15/04/08
TSS	11		30/05/08
TSS	14		30/05/08
TSS	11		26/06/08
TSS	3		23/07/08
TSS	9		11/08/08
TSS	7		18/08/08
TSS	7		25/08/08
TSS	3		15/09/08
TSS	5		18/09/08
TSS	0		22/09/08
TSS	4		29/09/08
TSS	37		10/10/08
TSS	41		30/10/08
TSS	6		30/10/08
TSS	6		3/11/08
TSS	3		3/11/08
TSS	10		6/11/08
TSS	49		6/11/08
TSS	9		10/11/08

Analyte	RPD (%)	Error (%)	Sampling Date
TSS	5		10/11/08
TSS	27		17/11/08
TSS	54		26/01/09
TSS	9		26/01/09
TSS	6		28/01/09
TSS	6		28/01/09
TSS	1		6/02/09
TSS	5		13/02/09
TSS	4		13/02/09
TSS	1		16/02/09
TSS	1		20/02/09
TSS	2		26/02/09
TSS	7		26/02/09
TSS	6		12/03/09
TSS	4		16/03/09
TSS	0		16/03/09
TSS	41		19/03/09
TSS	3		23/03/09
TSS	0		23/03/09
TSS	9		26/03/09
TSS	3		30/03/09
TSS	1		30/03/09
TSS	2		2/04/09
TSS	10		16/04/09
TSS	1		16/04/09
TSS	0		20/04/09
TSS	5		23/04/09
TSS	14		23/04/09
TSS	0		27/04/09
TSS	2		27/04/09
TSS	1		27/04/09
TSS	1		30/04/09
TSS	27		30/04/09
TSS	3		4/05/09
TSS	6		4/05/09
TSS	0		1/10/09
TSS	10		1/10/09
TSS	2		17/10/09
TSS	5		19/10/09
TSS	1		20/10/09
TSS	6		20/10/09
TSS	1		22/10/09
TSS	4		22/10/09
TSS	3		27/10/09
TSS	5		2/11/09
TSS	3		4/11/09
TSS	4		5/11/09
TSS	19		5/11/09
TSS	3		9/11/09
TSS	12		9/11/09
TSS	4		11/11/09
TSS	1		12/11/09
TSS	2		16/11/09
TSS	10		16/11/09
TSS	4		17/11/09
TSS	8		19/11/09

Shading indicates value outside QA/QC limit.

Table B-3. QA/QC Data of Samples, Standards, and Blanks.

Analyte (Range [mg/L])	RPD (%)	Error (%)	Sampling Date
TSS	1		23/11/09
TSS	7		25/11/09
TSS	5		26/11/09
TSS	5		30/11/09
TSS	2		1/12/09
TSS	7		3/12/09
TSS	4		7/12/09
TSS	7		7/12/09
TSS	23		10/12/09
TSS	3		15/12/09
TSS	1		23/12/09
TSS	6		26/12/09
TSS	4		28/12/09
TSS	2		30/12/09
TSS	35		31/12/09
TSS	0		4/01/10
TSS	0		6/01/10
TSS	16		6/01/10
TSS	7		8/01/10
TSS	1		11/01/10
TSS	8		13/01/10
TSS	7		13/01/10
TSS	13		21/01/10
TSS	4		26/01/10
TSS	11		28/01/10
TSS	11		28/01/10

Shading indicates value outside QA/QC limit.

Analyte	RPD (%)	Error (%)	Sampling Date
TSS	5		5/02/10
TSS	4		13/02/10
TSS	6		19/02/10
TSS	2		22/02/10
TSS	16		4/03/10
TSS	2		10/03/10
TSS	6		11/03/10
VS	11		27/03/08
VS	0		25/08/08
VS	46		25/08/08
VS	9		25/08/08
VS	6		25/08/08
VS	23		20/02/09
VS	13		20/02/09
VS	2		4/05/09
VSS	60		17/02/08
VSS	23		26/02/08
VSS	8		26/02/08
VSS	1		29/02/08
VSS	7		3/03/08
VSS	5		7/12/09
VSS	1		10/03/10
VSS	3		3/11/08
VSS	1		3/11/08
VSS	1		12/02/10

Appendix C. Supplementary Experimental Data

C.1 Preliminary Experiments (Chapter 3)

Table C-1. Data from AS Addition Experiment.

P6 TSS:	45	mg/L
P6 Temp:	23.7	C

AS TSS:	3,180	mg/L
AS SV:	450	mL/L
AS SVI:	141	mL/g

Test A	Jar	P6 Volume (L)	AS Added (mL)	Calc. P6 TSS (mg/L)	Calc. AS TSS (mg/L)	Calc. Mixture TSS (mg/L)	Mixing Speed (rpm)	Mixing Time (min)	Separation (S / F)	Separation Time (min)	Final TSS (mg/L)
Test A	C1a	1	0	45	0	45	25	20	S	30	34
Test A	C2a	1	3	45	10	55	25	20	S	30	38
Test A	C3a	1	16	45	50	95	25	20	S	30	45
Test A	C4a	1	31	44	96	140	25	20	S	30	42
Test A	C5a	1	63	43	188	231	25	20	S	30	34
Test A	C6a	1	126	40	356	396	25	20	S	30	30
Test B	C1b	1	0	45	0	45	25	1	S	30	44
Test B	C2b	1	3	45	10	55	25	1	S	30	44
Test B	C6b	1	126	40	356	396	25	1	S	30	46
Test C	C1c	1	0	45	0	45	25	20	F	1	45

Table C-2. Nitrogen Data from SBR Experiment.

Type	RP	TN (mg/L N)	Filtered TN (mg/L N)	NO3 (mg/L N)	Filtered NO3 (mg/L N)	NO2 (mg/L N)	NH3 (mg/L N)	Filtered NH3 (mg/L N)
M1	1	32	-	1.5	-	-	27.9	-
M1	2	38	-	2.0	-	-	25.4	-
M1	3	-	-	0.0	-	-	18.3	-
M1	4	-	-	0.0	-	-	21.5	-
M1	5	-	-	0.0	-	-	15.4	-
M1	6	-	-	1.0	-	-	12.1	-
M1	7	-	-	0.0	-	-	14.8	-
M1	8	-	-	1.2	-	<2	11.0	-
M1	9	-	-	-	0.6	-	22.5	16.8
M1	10	-	-	-	1.4	-	-	40.1
M1	11	-	52	-	1.2	-	-	45.5
M1	12	-	-	-	1.7	-	-	38.4
M1	13	-	-	-	1.1	-	-	40.6
M1	14	-	-	-	2	-	-	36.8
M3	1	26	-	0.3	-	-	15.0	-
M3	2	-	-	0.0	-	-	8.1	-
M3	3	-	-	0.0	-	-	5.8	-
M3	4	-	-	0.0	-	-	3.3	-
M3	5	-	-	0.0	-	-	7.4	-
M3	6	-	-	3.0	-	-	0.3	-
M3	7	-	-	0.0	-	<2	4.0	-
M3	8	-	-	0.0	-	-	0.1	-
M3	9	-	-	-	1.6	-	-	38.7
M3	10	-	57	-	0.9	-	-	53.2
M3	11	-	-	-	1.2	-	-	42.4
M3	12	-	-	-	1.3	-	-	46.6
M3	13	-	-	-	2	-	-	44.8
M3	14	105	36	-	0.3	-	-	31.8

Note: M1=day 1 mixture; M3=day 3 mixture; S3=day 3 supernatant; S12=day 12 supernatant.

C.2 Feed Water Selection Experiments (Chapter 4)

Table C-3. Inoculum Standardisation Data from Feed Water Selection Experiments.

		1-P6			Mixture			
Test	Culture Day	Turbidity (NTU)	TSS (mg/L)	Chl a (ug/L)	Turbidity (NTU)	TSS (mg/L)	Chl a (ug/L)	Notes
T2	4.0	7.7	18					
T2	6.9	10.8	27					
T2	7.9	18.5	53	491				
T2	8.9	15.7	40					
T2	9.9				23.4			standard mixed
T2	9.9				21.6	59	636	mixed + 6L tap water
T3	2.0	6.2						
T3	3.0	6.2						
T3	4.2	5.7	5	27				
T3	7.0	24.9	33	250				
T3	8.0	29.8	57					
T3	9.0	76.7	88					
T3	9.1				8.6	18		70L standard mixed + 30L tap water
T3	9.2				15.1	42		90L standard mixed + 30L tap water
T3	9.9				19.9	57	306	90L standard mixed + 30L tap water
T4	0.0		39					after 50% dilution of P6 (was 73 mg/L)
T4	1.8		73					
T4	4.7		69					
T4	4.8					71		mixed + 5L tap water
T4	4.9					71	333	mixed + 10L tap water
T5	0.0		52					then 50% dilution of P6 water
T5	2.7		36					
T5	3.7		64					
T5	4.7		79					
T5	5.7		108					
T5	5.8					57	395	48L standard + 40L tap water
T6	0.0		61					P6 water
T6	0.1		36					P6 water + 30% dilution
T6	4.1		87					
T6	4.9		83					
T6	5.9		100					
T6	6.0					73	574	14L standard + 7L tap water / SBR

C.2.1 Difficulties Encountered

Electrical problems caused random power interruptions to reset the computer controlling the sequence of the SBRs often resulting in substantial downtime (Table C-4). During downtime, SBRs were essentially in settle period only–receiving no light or mixing. Dedicated troubleshooting was performed, and several “solutions” were trialled including use of different outlets within the room, installation of a noise suppressor, more frequent de-icing of the cooling coil to reduce electrical load on the circuit, replacement of surge-protected power boards, and others to no avail. Eventually, an uninterruptible power supply (UPS) was installed and a faulty gas monitor was bypassed at the start of T3. No further electrical problems were experienced.

Table C-4. Power and Memory Faults Experienced Causing Downtime.

Test Run	Day Computer Failed	Day Computer Restarted	Cause of Failure	Downtime (h)	Run Total Downtime (h)
T1	7.9	8.0	Power	3.4	66.6
	8.2	8.2	Power	1.4	
	9.8	12.0	Power	51.2	
	16.7	17.1	Power	10.6	
T2	-12.4	-12	Power	9.0	29.6
	-2.7	-1.9	Power	15.3	
	1.0	1.0	Power	0.3	
	2.0	2.0	Power	0.1	
	2.9	3.1	Power	4.9	
T3	-14.9	-14.9	Power	0.0*	4.0
	4.9	5.1	Computer	4.0	
T4	4.1	7.0	Computer	42.4	42.4
T5	-3.7	-3.0	Computer	17.6	17.6
T6	-10.8	-10.0	Computer	19.7	19.7

Note: (-) test day = failure occurred during acclimation period; (*) = UPS prevented shutdown; power = settle period only during downtime; computer = dark and mix period only during downtime.

Once the power issue was fixed, however, computer failures emerged at longer run times (i.e., since the computer was no longer reset irregularly due to electrical problems) beginning with T3 (Table C-4). These failures occurred during the dark and mix period and prevented progression to the settle and drain steps until the computer was reset after every failure. The computer was subsequently replaced and reset weekly to prevent memory faults beginning with T6.

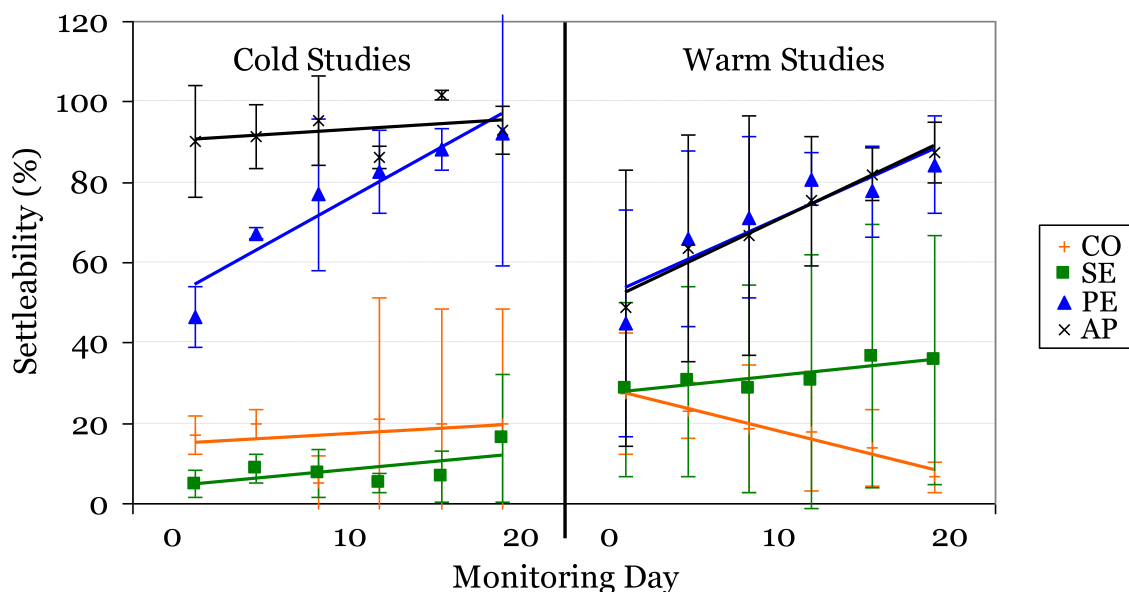

Figure C-1. Settleability of Microbial Cultures over Time during Feed Water Selection Experiments.

Table C-5. Heat of Combustion Data from Feed Water Selection and Preliminary SBR Experiments.

Reactor	Test	Sample	Value (MJ/kg)	*M* ± *SD* (MJ/kg)
AP Cold Studies	T1-3	1	25.0	24.8 ± 0.3
AP Cold Studies	T1-3	2	24.5	
AP Warm Studies	T4-6	1	25.6	24.9 ± 0.9
AP Warm Studies	T4-6	2	24.3	
PE Cold Studies	T1-3	1	21.8	21.6 ± 0.4
PE Cold Studies	T1-3	2	21.3	
PE Warm Studies	T4-6	1	27.1	25.7 ± 2.0
PE Warm Studies	T4-6	2	24.3	
SBR1/2	-	1	25.0	24.6 ± 0.4
SBR1/2	-	2	24.3	
SBR1/2	-	3	24.3	

C.3 Retention Time Experiments (Chapter 5)

Table C-6. Inoculum Standardisation Data from Retention Time Experiments.

Test	Culture Day	Temp (C)	RH (%)	P6 TSS (mg/L)	Notes
T1	0.0	16.4	60	20	
T1	0.8	11.1	60	32	
T1	1.9	14.1	58	52	
T1	2.8	14.4	65	90	
T1	2.9			67	12.6 L P6 + 4.9 L tap mixed inoculum
T2	0.0	19.0		28	
T2	0.8	12.1		44	
T2	0.9			33	14 L P6 diluted to 25.5 L mixed culture
T2	3.8	11.4	65	38	
T2	4.0			45	
T2	4.7	11.3	65	52	
T2	4.8			66	

Table C-7. Inoculum Characteristics from Retention Time Experiments.

	TSS (mg/L)	Chl a (mg/L)	TP (mg/L)	TKN (mg/L)	TC (mg/L)	TOC (mg/L)	SVI (mL/g)
P6 water	67 ± 0.8	0.4 ± 0.0	2.6 ± 1.6	14.5 ± 3.5	51 ± 8	26 ± 10	–
Activated sludge	4,515 ± 198	0.5 ± 0.4	97 ± 2	465 ± 7	1,471 ± 62	1,045 ± 377	154 ± 10
Primary wastewater	83 ± 25	0.0 ± 0.0	–	–	129 ± 36	111 ± 22	–
Inoculum	161 ± 1	0.3 ± 0.0	5.0 ± 1.3	28.8 ± 3.1	92 ± 3	62 ± 19	90 ± 7

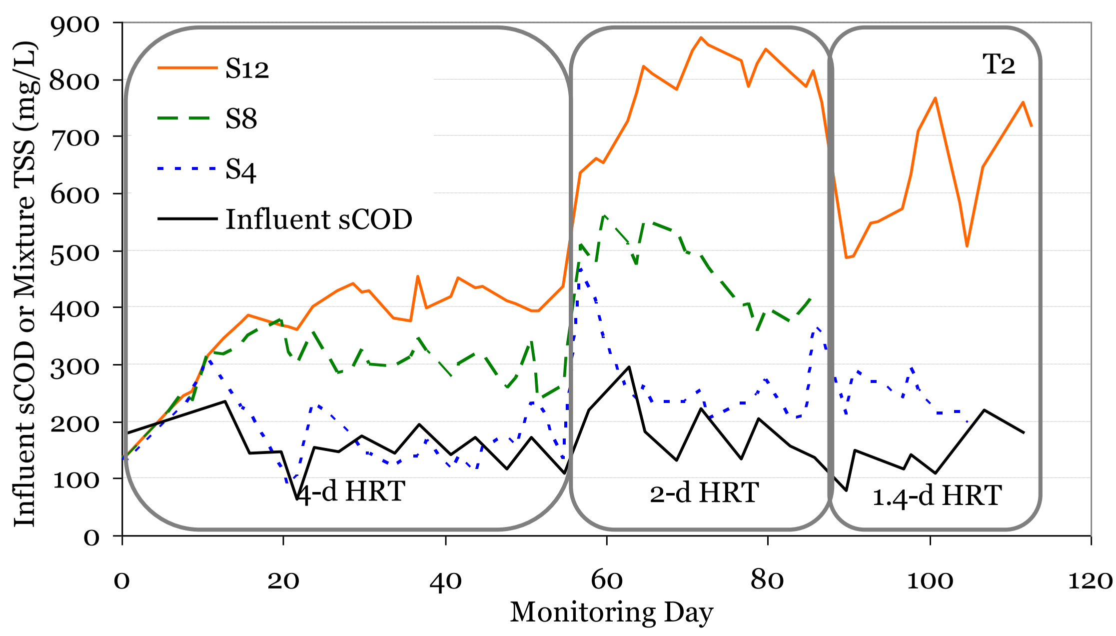

Figure C-2. Influent sCOD and Mixture TSS of Microbial SBRs Operated at Different HRTs during Feed Water Selection Experiments.

Table C-8. TSS and COD Contents of Microbial SBRs during Retention Time Experiments.

SBR	Monitoring Day	TSS (mg/L)	COD (mg/L)	COD/TSS Ratio
S12	14	391	605	1.55
S12	19	424	674	1.59
S12	28	433	675	1.56
S12	35	475	697	1.47
S12	42	511	675	1.32
S12	49	467	687	1.47
S12	16	386	637	1.65
S12	22	361	600	1.66
S12	30	426	673	1.58
S12	37	455	556	1.22
S12	51	393	578	1.47
S12	63	725	1222	1.68
S12	72	871	1384	1.59
S12	79	827	1422	1.72
S12	86	814	1336	1.64
S12	101	766	1136	1.48
S8	14	369	525	1.42
S8	19	381	592	1.55
S8	28	389	559	1.44
S8	35	318	443	1.39
S8	49	245	293	1.20
S8	16	349	573	1.64
S8	22	299	517	1.73
S8	30	325	530	1.63
S8	37	345	439	1.27
S8	51	346	419	1.21
S8	63	511	819	1.60
S8	72	488	832	1.70
S8	79	357	527	1.48
S8	86	423	691	1.63
S4	14	301	451	1.50
S4	19	190	317	1.66
S4	28	168	287	1.71
S4	35	178	303	1.70
S4	42	261	287	1.10
S4	49	153	281	1.84
S4	16	217	467	2.15
S4	22	104	220	2.12
S4	30	144	272	1.89
S4	37	140	204	1.46
S4	51	233	385	1.65
S4	63	252	581	2.31
S4	72	254	317	1.25
S4	79	248	259	1.04
S4	101	215	427	1.99