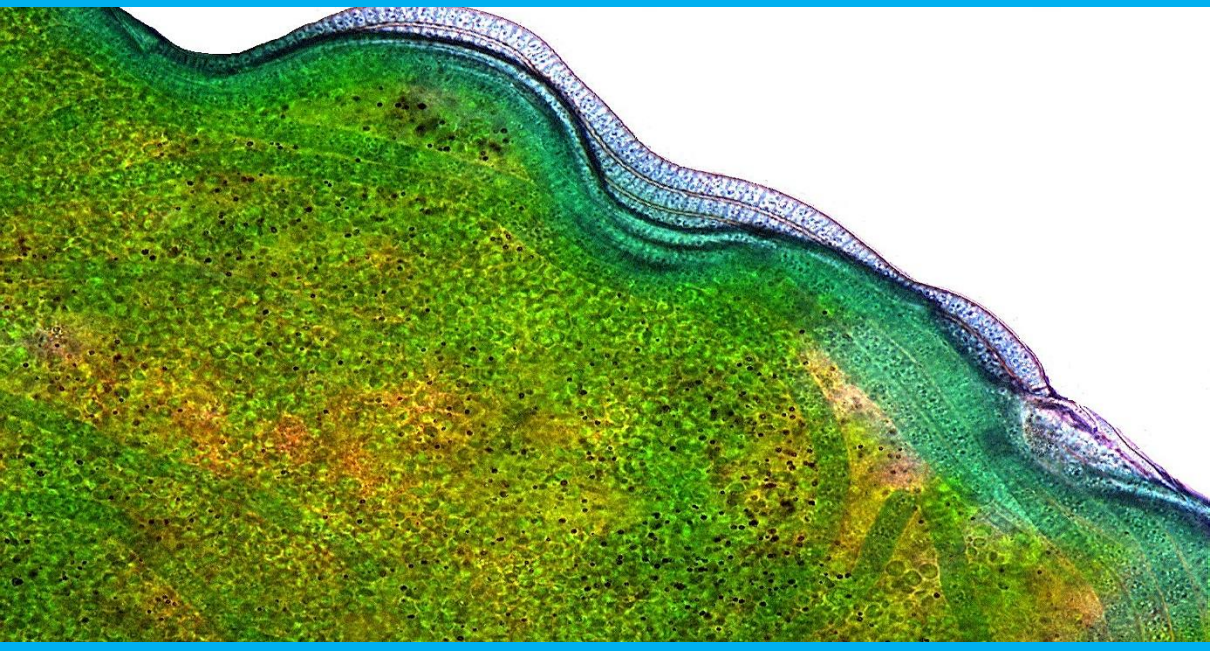

Microalgal bacterial flocs for wastewater treatment: from concept to pilot scale

Sofie Van Den Hende

UNIVERSITEIT GENT

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

The stone which the builders rejected,

the same is become the head of the corner

(Matthew 21:42)

Promotors

Prof. Dr. ir. Nico Boon	Laboratory of Microbial Ecology and Technology (LabMET), Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium
Dr. ir. Han Vervaeren	Laboratory of Water and Eco-technology (LIWET), Department of Industrial Biological Sciences, Faculty of Bioscience Engineering, Ghent University, Kortrijk, Belgium

Examination committee members

PD. Dr. res. nat. Anneliese Ernst	Institut für Physikalische Prozesstechnik, Fakultät für Ingenieurswissenschafte, Hochshule für Technik und Wirtschaft des Saarlandes (HTWds), Saarbrücken, Germany
Prof. Dr. ir. Patrick Sorgeloos	Laboratory of Aquaculture and Artemia Reference Center, Department of Animal Production, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium
Prof. Dr. ir. Peter Goethals	Laboratory of Environmental Technology and Aquatic Ecology, Department of Applied Ecology and Environmental Biology, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium
Dr. ir. Jean-Philippe Steyer	Laboratoire de Biotechnologie et de l'Environnement (LBE), INRA, Narbonne, France
Prof. Dr. ir. Peter Bossier (Secretary)	Laboratory of Aquaculture and Artemia Reference Center, Department of Animal Production, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium
Prof. Dr. ir. F. Devlieghere (Chairman)	Laboratory of Food Microbiology and Food Preservation (LFMFP), Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium

Dean

Prof. Dr. ir. Guido Van Huylenbroeck	Faculty of Bioscience Engineering, Ghent University, Gent, Belgium

Rector

Prof. Dr. Anne De Paepe	Ghent University, Gent, Belgium

Microalgal bacterial flocs to treat wastewater: from concept to pilot scale

Sofie Van Den Hende

Thesis submitted in fulfillment of the requirements

for the degree of Doctor (Ph.D.) in Applied Biological Sciences

Dutch translation of the title

Microalgenbacteriënvlokken voor behandeling van afvalwater: van concept tot pilootschaal

Cover illustrations

Cover front: Part of a microalgal bacterial floc (MaB-floc) from a sequencing batch reactor treating paper mill wastewater (optical bright field microscopy). Illustration details are presented in Chapter 5.

Cover back: Overview of input and output of an outdoor MaB-floc raceway pond on pilot scale. Illustration details are presented in Chapter 1.

Funding

This work was funded by an assistant Ph.D. scholarship from Howest University College and Ghent University, by the EnAlgae project with financial support from the INTERREG IVB NWE program, Flemish Government and the Province West-Flanders, and by a Ph.D. award from the Ernest du Bois Fund managed by King Baudouin Foundation.

ISBN

978-90-5989-684-0

To refer to this work

Van Den Hende, S., 2014. Microalgal bacterial flocs for wastewater treatment: from concept to pilot scale. Ph.D. dissertation. Ghent University, Ghent, Belgium.

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Notation index

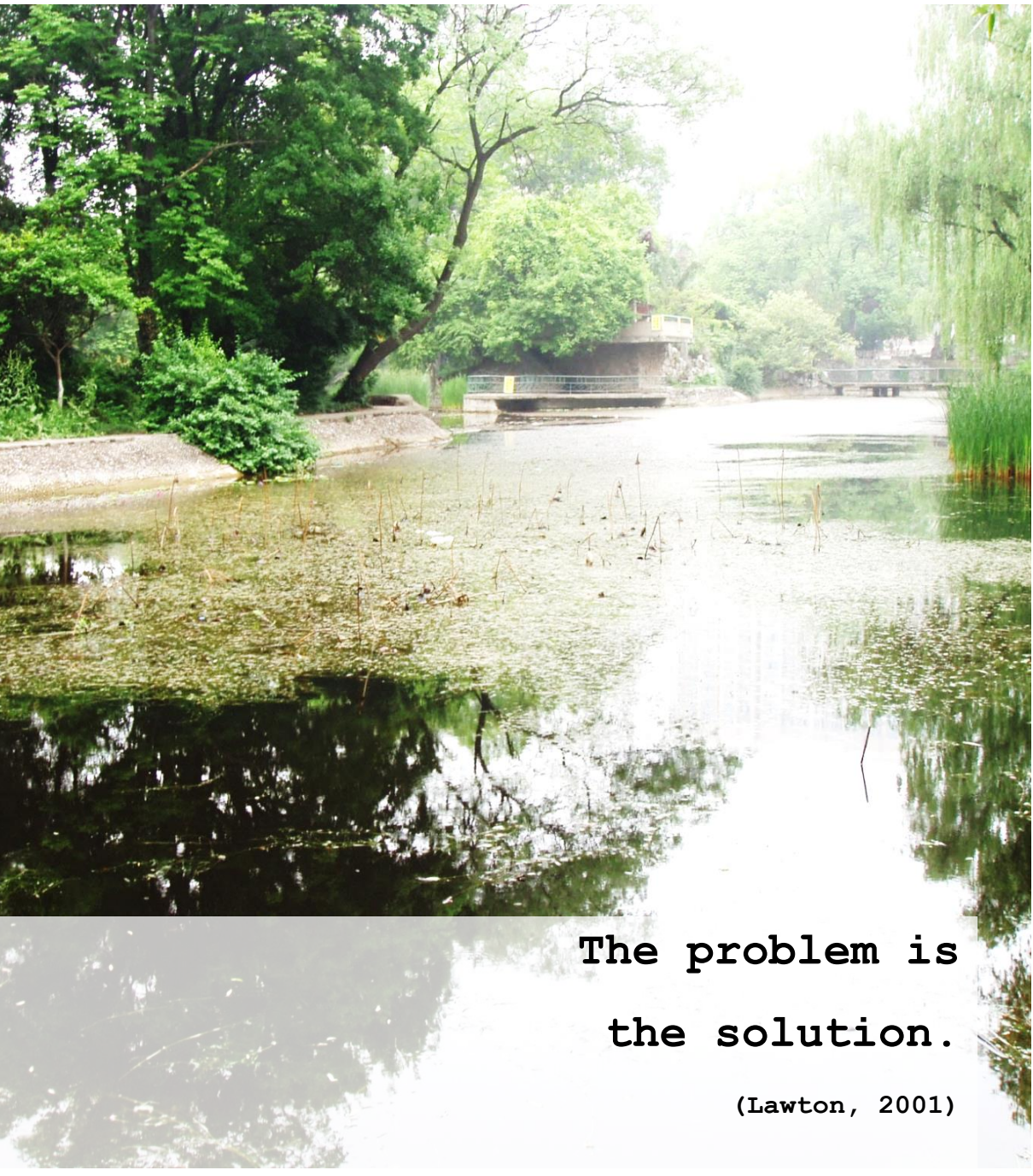
Abbreviations

AI	autotrophic index
ATP	adenosine triphosphate
CA	carbonic anhydrase
CAS	conventional activated sludge system
CSTR	continuous stirred tank reactor
C_xH_y	carbohydrates
EPS	extracellular polymeric substances
FTU	formazine turbidity unit
HRAP	high-rate algal pond
IC	ion chromatograph
MaB-floc	microalgal bacterial floc
MaB-S-WWT	microalgal bacterial system for wastewater treatment
$NADPH_2$	nicotinamide adenine dinucleotide phosphate
NTU	nephelometric turbidity unit
PAR	photosynthetic active radiation
PM	photosynthetic microorganism
PSI / II	photosystem I / II
rpm	rotations per minute
rubisco	ribulose-1,5-biphosphate carboxylase oxygenase
SBR	sequencing batch reactor
SEM	scanning electron microscopy
UASB	upflow anaerobic sludge blanket
VFA	volatile fatty acids
WSP	wastewater stabilization ponds
WWTP	wastewater treatment plant

Parameters and units

BOD_5	biological oxygen demand	(mg O_2 L^{-1})
Chl*a*	chlorophyll *a*	(mg L^{-1})
CO	carbon monoxide	(g Nm^{-3})
CO_2	carbon dioxide	(g Nm^{-3})

COD	chemical oxygen demand	(mg O_2 L^{-1})
DisCa	dissolved calcium	(mg Ca L^{-1})
DO	dissolved oxygen	(mg O_2 L^{-1})
DM	dry matter	(%DM)
dPPFD	daily photosynthetic active photon flux density	(mmol PAR photons $L_{reactor}^{-1}$ day^{-1})
dSVI	diluted sludge volume index	(mL g^{-1} TSS)
HRT	hydraulic retention time	(days)
$K_{m(CO2)}$	half saturation constant of CO_2	(µmol L^{-1})
NO	nitrogen oxide gas	(g Nm^{-3})
NO_2	nitrogen dioxide gas	(g Nm^{-3})
NO_3^--N	nitrate nitrogen	(mg N L^{-1}; g N L^{-1})
NO_2^--N	nitrite nitrogen	(mg N L^{-1}; g N L^{-1})
NO_x	nitrogen oxides	(g Nm^{-3})
PO_4^{3-}-P	ortho-phosphate-phosphorous	(mg P L^{-1}; g P L^{-1})
PPFD	photosynthetic active photon flux density	(µmol PAR photons m^{-2} s^{-1})
RE	removal efficiency	(%)
RR	removal rate	(mg $L_{reactor}^{-1}$ day^{-1})
SCOD	soluble chemical oxygen demand	(mg O_2 L^{-1})
SO_2	sulfur dioxide	(g Nm^{-3})
SO_x	sulfur oxides	(g Nm^{-3})
SRT	sludge retention time	(days)
SVI	sludge volume index	(ml g^{-1} TSS)
TC	total carbon	(mg C L^{-1})
TCa	total calcium	(mg Ca L^{-1})
TIC	total inorganic carbon	(mg C L^{-1})
TN	total nitrogen	(mg N L^{-1})
TOC	total organic carbon	(mg C L^{-1})
TP	total phosphorous	(mg P L^{-1})
TSS	total suspended solids	(mg or g TSS L^{-1})
VSS	volatile suspended solids	(mg or g VSS L^{-1})



The problem is

the solution.

(Lawton, 2001)

Picture on previous page:

Eutrophication of surface water resulting in algal growth, Xi'An, China.

CHAPTER 1

Introduction and thesis outline

1.1. General introduction

Water is indispensable for life on earth. After its use for a variety of human applications, its quality degenerates to a level such that if it was disposed to any water-receiving body without treatment, there would be damage to human health and the environment (Elias, 2014). This used water, termed wastewater, contains pollutants/contaminants which are to be removed before disposal to natural waters. Decades ago, wastewaters were routinely discharged to natural waters for purification (Graham and Wilcox, 2000). This discharge caused an increase in nutrients of natural waters, termed eutrophication ('eutrophos / ευτροφοσ' is Greek for 'well-nourished') and led to undesired algal blooms. Later on, as population densities and environmental awareness increased, wastewater treatment plants were built. The primary goals of these plants were to protect downstream users (Wilsenach et al., 2003) and to avoid eutrophication of natural waters (Tchobanoglous et al., 2003).

With principles of sustainability gaining greater value in all fields of engineering, conventional wastewater treatment systems solely focusing on pollutant removal are facing increasing scrutiny (Henze et al., 2008). World issues regarding depletion of resources and global warming, call for a wastewater treatment with nutrient recovery and decreased greenhouse gas emissions. Although water sanitation dates back from Mesopotamian times (Lofrano and Brown, 2010), the ultimate wastewater treatment system is yet to be designed. Microalgae may play a key role in this redesign. Being photosynthetic organisms, microalgae can lower the emission of CO_2, lower the need for mechanical aeration, scavenge resources from the wastewater and convert solar energy into biomass. By doing so, the problem of eutrophication coupled with algal blooms may be converted into a solution.

In this chapter, features and limitations of conventional wastewater treatment systems in Northwest Europe are briefly reviewed to evidence the potential for sunlight-powered microalgal bacterial systems for wastewater treatment (MaB-S-WWT). Particular emphasis is given to oxygenic photosynthesis and the key factors for photosynthetic microalgal growth. Furthermore, features and challenges of MaB-S-WWT and biomass production are discussed. One of the major burdens hampering the implementation of MaB-S-WWT is the high cost for microalgae harvesting. Bioflocculation of microalgae and bacteria is presented as a way to address this burden. Finally, the objectives and outline of this dissertation are defined.

1.2. Conventional wastewater treatment in Northwest Europe

Nowadays, there are is a wide variety of systems which can be applied for wastewater treatment (Henze et al., 2008). In general, several wastewater treatment processes (Table 1.1.) are being applied in series to reach target norms for certain parameters, such as biochemical oxygen demand (BOD_5), nitrogen (N) and phosphorous (P). In general, the most critical aspects in the choice of a wastewater treatment system in industrialized countries in Northwest Europe are nutrient removal efficiency, reliability, land requirements and sludge disposal (von Sperling, 1996). Therefore, waste stabilization ponds, worldwide one of the most frequently applied treatment system, is limited in Northwest Europe (von Sperling, 1996; Shilton, 2005).

1.2.1. Features

In most conventional plants for treatment of (agro)-industrial wastewater and sewage in Belgium, the treatment involves 3 steps. During a primary treatment, settleable or floating solids are mechanically removed from the raw wastewater by screen, grit chambers, sedimentation and air flotation (Tchobanoglous et al., 2003). Secondary treatment removes suspended and dissolved organic matter. In high BOD_5 loaded wastewater, an anaerobic reactor, such as an upflow anaerobic sludge blanket reactor (UASB), converts organic carbon to biogas enabling energy recovery. Anaerobic digestion comprises hydrolysis, acidogenesis, acetogenesis and methanogenesis (Henze et al., 2008). The resulting UASB effluent or low BOD_5 loaded wastewater is treated in a conventional activated sludge system (CAS). In this system, BOD_5 is oxidized by aerobic bacteria to CO_2, H_2O and biomass. To provide aerobic conditions, reactors are mechanically aerated. Next to this metabolic process, decay and removal via flocculation play a role in CAS (Cervantes et al., 2006).

Next to CAS systems, also bacterial biofilms are used, such as trickling filters and rotating biological contactors (Tchobanoglous et al., 2003). To remove nitrogen compounds, sequential processes of nitrification and denitrification are applied in activated sludge processes. Nitrification is performed by strictly aerobic microorganisms using O_2 as electron acceptors (Henze et al., 2008):

$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+$ nitritation (E.1.1.)

$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$ nitratation (E.1.2.)

Nitritation is performed by ammonia oxidizing bacteria (AOB), e.g., of the genera *Nitrosomonas*. Nitrite oxidizing bacteria (NOB), e.g., of the genera *Nitrospira*, perform nitratation. During denitrification, which takes place in an anoxic environment, nitrate is the electron receptor and is reduced to NO_2^-, NO, N_2O and finally N_2 (Tchobanoglous et al., 2003). This reaction is fuelled by organic carbon as electron donor, for example (Henze et al., 2008):

NO_3^- + 4 g COD + H^+ -> 0.5 N_2 + 1.5 g sludge (E.1.3.)

Nitrification and denitrification have a net oxygen consumption of 1.7 mg oxygen per mg nitrogen (Henze et al., 2008). Also ammonium assimilation and ammonification, i.e. conversion of organic nitrogen to ammonium, takes place in activated sludge (Cervantes et al., 2006). Novel technologies of nitrogen removal include anoxic ammonium oxidation (anammox) and its numerous variants (Henze et al., 2008).

Table 1.1. *Overview of conventional wastewater treatment systems for removal of organic matter and nutrients*
Based on von Sperling, 2006; Shilton, 2005.

System	**Removal efficiency (%)**					**Cost[2]**
	BOD_5	**NH_3**	**NH_4^+ ->NO_3^-**	**NO_3^- -> N_2**	**TP**	**(€ m^{-3})**
Mechanical treatment	20-35	-[1]	-	-	-	0.02-0.06
Aerated activated sludge	60-90	-	-	-	10-45	0.19-0.30
Flocculation	40-60	-	-	-	30-60	0.05-0.07
Chemical precipitation	50-70	-	-	-	65-95	0.08-0.14
Ammonia stripping	-	70-95	-	-	-	0.19-0.30
Nitrification	-	-	80-95	-	-	0.15-0.23
Denitrification	-	-	-	70-90	-	0.11-0.19
WSP[3]	70-90		20-70[4]		20-60	0.02-0.06

[1] Removal of this compound is not aimed at; [2] Cost per volume wastewater based on 0.75 € per $; [3] Waste stabilization pond including anaerobic pond; [4] Total nitrogen removal.

1.2.2. Challenges

In a typical wastewater treatment plant employing a CAS, up to 45-75 % of the total energy consumption of 1.1-2.4 MJ m^{-3} wastewater is due to activated sludge aeration (Henze et al., 2008; Tchobanoglous, 2003). Moreover, this mechanical aeration is limited by the poor aqueous solubility of oxygen and may cause pathogen spray and hazardous volatilization of organic molecules (Bell et al., 1993).

In the recent years, concern has increased about the greenhouse gas emissions from wastewater treatment plants (WWTPs). In WWTPs, the greenhouse gasses CO_2, N_2O and CH_4 are produced during microbial wastewater treatment, while CO_2 and CH_4 are indirectly emitted from the electricity demands of these treatment plants (Corominas et al., 2013). CAS processes emit around 1.5 kg CO_2 per kg COD treated, and around 5 kg CO_2 per kg N removed (Henze et al., 2008). Moreover, during denitrification processes, N_2O has been observed as main end product over N_2 (Zeng et al., 2003). CH_4 and N_2O are greenhouse gasses with a global warming potential of 25 and 298 times that of CO_2, respectively (Solomo et al., 2007).

Anammox processes and its variants can significantly lower the CO_2 emissions, but, similar as in nitrification/denitrification processes, still aim at fast nitrogen removal instead of nitrogen recovery (Henze et al., 2008). Moreover, practical constraints hinder the wide application of anammox processes (Vilar et al., 2010). Nitrogen fixation from N_2 into NO_3^-, NH_3 or urea costs energy. So, from the ecological point of view it seems beneficial to recover these nutrients directly from the wastewater instead of converting them first into N_2 to lateron convert them again into nitrogen fertilizer.

As for P, its chemical removal results in flocculant-rich sludge causing secondary contamination (Smil, 2000). Discharge norms for sulphates and chlorides are becoming more severe and limit chemical P removal. Moreover, in some sensitive water bodies, P discharge limits below 0.1 mg L^{-1} have been promulgated (Henze et al., 2008). To achieve such levels, coagulants combined with filtration or ultrafiltration are needed, resulting in considerable expenses (Henze et al., 2008).

In conclusion, conventional wastewater technologies are end-of-pipe technologies focusing on an efficient removal rather than resource-recovery, often at the expense of global warming (Cervantes et al., 2006). A paradigm shift from waste to resource recovery is calling (Corominas et al., 2013).

1.3. Sunlight-powered microalgal bacterial systems for wastewater treatment

To address the challenges of the CAS system, heterotrophic bacteria in these systems need a 'microbial partner' which can produce oxygen *in situ* powered by an infinite energy source and which can scavenge CO_2 and nutrients from the wastewater. In this way, oxygenic microalgae seem the perfect match. The resulting sunlight-powered microalgal bacterial systems for wastewater treatment (MaB-S-WWT) have large potential to provide an energy-efficient and nutrient-recovering system. However, the amount of papers regarding these systems where microalgal bacterial biomass is recovered largely outnumbers the amount of industrial applications in Northwest Europe. Therefore, key processes, factors, interactions and reactor performances of MaB-S-WWTs, with focus on microalgae, are outlined in order to identify their major challenges. Furthermore, the most frequently applied MaB-S-WWT is compared to the CAS system.

1.3.1. Oxygenic photosynthesis

Photosynthesis represents a unique process in which sunlight energy is used to extract protons and electrons from a variety of electron donor molecules, such as H_2S or H_2O, to reduce CO_2 to form organic molecules (Raven et al., 1992). This thesis focusses on oxygenic photosynthesis with the overall reaction:

$$6\ CO_2 + 12\ H_2\mathbf{O} + 8\ \text{photons} \rightarrow C_6H_{12}O_6 + 6\ \mathbf{O_2} + 6\ H_2O \qquad \text{(E.1.4.)}$$

Oxygenic photosynthetis can be divided into two closely linked processes: the light-dependent reactions and the light-independent reactions (Falkowsky and Raven, 2007; Richmond, 2004; Carvalho et al., 2001; Raven et al., 1992). The light-dependent reactions occur in the thylakoid membranes. Two large protein complexes, named photosystems, can be distinguished (Fig. 1.1). Photosystem I (PSI) contains reaction center P700 and uses photons of 700 nm. Photosystem II contains reaction center P680 and uses photons of 680 nm. Upon illumination, light is absorbed by photosynthetic antennae (pigments) and its energy is transferred to P680 and P700, resulting in excitation of electrons. The excitated electrons are transferred through a chain of electron carriers to reduce nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate dihydrogen ($NADPH_2$). Simultanously, protons are transported from the stroma into the intrathylakoid space

forming a pH gradient. This gradient drives adenosine triphosphate (ATP) synthesis from phosphate groups (Pi) via ATP synthase during photophosphorylation. The electrons lost in PSII and PSI are replaced by splitting water. The overall equation for the light-dependent reactions is (Raven et al., 1992):

$2\ H_2O + 2\ NADP^+ + 3\ ADP + 3\ Pi + 8\ photons \rightarrow 2\ NADPH + 2\ H^+ + 3\ ATP$ (E.1.5.)

Certain photosynthetic eukaryotes can also perform cyclic photophosphorylation in which electrons cycle in a closed system around PS I and ATP is the only product.

The light-independent or dark reactions occur in the stroma (Fig.1.1.). The initial reaction is catalyzed by the enzyme ribulose-1,5-bisposphate carboxylase oxygenase (rubisco). Although some algal species, e.g., diatoms, are known to fix CO_2 via the C_4 or Hatch-Slack cycle (Reinfelder et al., 2000), most algae employ carbon fixation via the Benson–Calvin or C_3 cycle with intermediate 3-phosphoglycerate (PGA) containing 3 C molecules. The overall equation for the dark reactions is:

$6\ CO_2 + 12\ H^+ + 12\ NADPH^+\ 18\ ATP$

$\rightarrow C_6H_{12}O_6 + 12\ NADP^+ + 18\ ADP + 18\ Pi + 6\ H_2O$ (E.1.6.)

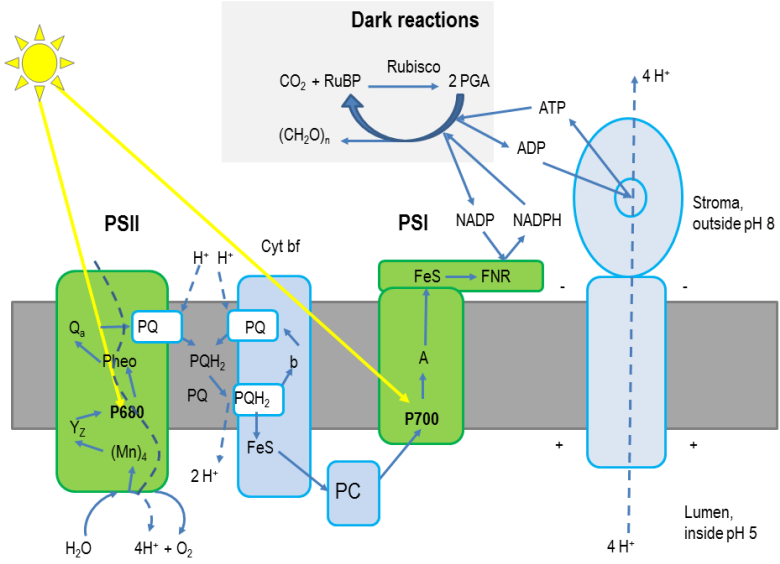


Fig. 1.1. *Shematic overview of oxygenic photosynthesis: light-dependent reactions and dark reactions of the Calvin-Benson cycle*

Electron transport chain is indicated by solid arrows; proton transport with dashed lines. Abbreviatons not mentioned in the text: PC: plastocyanine; PQ: plastoquinone; Cyt bf: cytochrome b6f complex; Pheo: pheophytina; Q_a: bound plastoquinone; PQH_2: free plastoquinol; A: phylloquinone; FNR: ferredoxin/NADP oxidoreductase; +/−: polarity of electric potential difference in light. Adapted from Falkowsky and Raven, 2007; Richmond, 2004.

1.3.2. Microalgae

The organisms commonly regarded as algae range from single-cell microalgal species to multi-cellular giant bladder kelp species. Both aquatic and non-aquatic species exist. In contrast to plants, algae lack organ structures and vascular tissues (Raven et al., 1992). Eukaryotic algae belong to the phyla Chlorophyta, Cryptophyta, Dinophyta, Euglenaophyta, Glaucophyta, Haptophyta, Ochrophyta (including Diatomea), Rhodophyta, while prokaryotic algae belong to the phylum Cyanobacteria (Graham and Wilcox, 2000). Prokaryotic algae do not contain the typical eukaryotic flagella and organelles (chloroplasts, mitochondria and nuclei) (Graham and Wilcox, 2000). All photosynthetic algae possess the photosynthetic pigment chlorophyll *a* (Chl*a*) (Richmond, 2004).

Unfortunately, possession of Chl*a* along with the ability to perform photosynthesis, does not characterize all of the organisms that are considered to be algae (Graham and Wilcox, 2000). Certain algae have a metabolic strategy different from photosynthesis. Next to obligate photoautotrophs, also heterotrophic algal species exist (Markou and Georgakakis, 2011). The latter obtain organic carbon from the external environment either by ingesting particles by a process known as phagotrophy, or through uptake of dissolved organic compounds, termed osmotrophy (Graham and Wilcox, 2000). Photoauxotrophic or obligate mixotrophic algae are incapable of synthetizing certain essential vitamins (biotin, thiamine and vitamin B_{12}). Numerous algae are facultative mixotrophs, i.e. can grow either by phototrophy or heterotrophy depending on the availability of light and organic carbon (Graham and Wilcox, 2000).

To ease reading, in this dissertation, the term microalgae is used for both eukaryotic and prokaryotic microscopic algae, regardless their nutritional strategy.

1.3.3. Factors influencing photosynthetic algal growth

Sunlight as energy source

Photosynthetis is strongly dependent of the quality and quantity of light. The light quality depends on the wavelength λ (m) of the photons (Markou and Georgakakis, 2011). The energy E (J) of a photon is given by $E = hc / \lambda$, where h is Planck's constant (6.626×10^{34} J s) and c is the speed of light in vacuum (3×10^{8} m s^{-1}) (Richmond, 2004). Photons with a wavelength below 400 nm have a high energy

and bring about ionizing effects in algae, whereas photons with a wavelength above 700 nm have a too low energy content for photosynthesis (Carvalho et al., 2011). Therefore, generally photosynthetically active radiation (PAR) ranges from 400 nm to 700 nm. However, not all these wavelengths can be used by microalgae. The light wavelengths absorbed by microalgae, depend on the photosynthetic pigments they contain. All chlorophyll pigments (*a*, *b*, *c*, *d*, *e*) absorb in the blue and red light region (450-475 nm and 630-700 nm), while carotenoids absorb in the blue light region (400-500 nm) and phycobilins absorb the orange-red light region (400-500 nm) (Markou and Georgakakis, 2011). Recently, a chlorophyll molecule (termed Chl*f*) was found that extends photosynthesis further into the infrared region (720 nm) (Chen et al., 2010). Only cyanobacteria and red algae contain phycobilins.

The light quantity is expressed as photosynthetic photon flux density (PPFD). The photosynthesis rate of microalgae is enhanced by increasing the PPFD up to the point of photo-saturation (Carvalho et al., 2011; Markou and Georgakakis, 2011). Photo-saturation is due to the slow catalytic rate of rubisco and cytochrome b6f (Richmond, 2004). Cytochrome b6f is an enzyme which transfers electrons between PSII and PSI (Fig. 1.1.). During photo-saturation, the excessive PPFD is dissipated as heat and fluorescence (Richmond, 2004). When excessive PPFDs become injurious, denoted as photo-inhibition, photosynthesis rates decrease (Fig. 1.2). Ultimately, algal cells are damaged due to production of excitated oxygen damaging PS II (Barsanti and Gualtari, 2006). This can eventually lead to algae death. Photo-saturation and photo-inhibition depend mainly on temperature, light source, algal species, strain and reactor type (Markou and Georgakakis, 2011). Most microalgae show photo-saturation at PPFD levels above 200 µmol PAR photons m^{-2} s^{-1} (Torzillo et al., 2003; Ogbanna and Tanak, 2000), but some species can tolerate 5000 µmol PAR photons m^{-2} s^{-1} (Richmond, 2004). Certain algal species can protect themselves to a certain extent to high PPFDs via photo-acclimation, hereby decreasing the amount and regulating the size of pigments (MacIntyre et al., 2002). Moreover, reactor conditions favouring biomass growth, such as optimal temperature and nutrient availability, increase the light tolerance (Richmond, 2004). Next to PPFD, also photoperiod determines light quantity. The provision of continuous light decreases the photosynthetic activity of some microalgal species (Becker, 2008).

Since photosynthesis requires 8 photons of 680 nm or 1408 kJ to fix one mol of 'CH_2O' (glucose) or 468 kJ, the photosynthetic solar light conversion efficiency is around 33% (Park et al., 2011b). However, only around 48 % of the direct solar radiation is PAR (Melis et al. 2009). In temperate climates, solar light reaches values above photo-saturation; in summer up to 2000 and in winter up to 1200 µmol PAR photons m^{-2} s^{-1} in winter are possible (Park et al., 2011b). Due to photo-saturation, most microalgal species in outdoor reactors only use 10 % (summer) to 17 % (winter) of this solar PAR light (Torzillo et al., 2003). Moreover, 10-20 % of PAR is lost by surface reflection (Park et al., 2011b). This leads to a maximum algal photosynthetic conversion efficiency of 1.3-2.4 % of the total solar radiation.

Oxygen inhibition

Dissolved oxygen (DO) levels reaching supersaturation can impact algal productivity (Molina et al., 2001). Oxygen competes with CO_2 at rubisco, hence initiating photorespiration (Richmond, 2004). During photorespiration, rubisco functions as oxygenase, catalyzing the reaction of O_2 with ribulose biphosphate to form phosphoglycolate. After dephosphorylation, glycolate is converted, in several steps, to CO_2, serine and ammonia, without any metabolic gain. While for most species, photorespiration is only 10 % of net photosynthesis, in some species it may be as high as 50 % (Richmond, 2004). By increasing the CO_2:O_2 ratio, the effects of photorespiration can be diminished (Richmond, 2004).

Temperature

The algal productivity depends on the temperature. Above the optimum temperature, increased dark respiration and photorespiration decrease the overall algal productivity (Park et al., 2011b). For many species the optimum temperature ranges between 28 °C and 35 °C (Soeder et al., 1995), while thermophilic species grow well at 42 °C (Wang et al., 2008a). The optimum temperature for photosynthetic growth can differ in light- and nutrient-limiting conditions (Park et al., 2011b). Since algae convert a large fraction of the sunlight into heat, excessive temperatures can occur at high PPFD and high biomass density (Richmond, 2004). Temperature can alter the water ionic equilibria and gas solubility (Jensen, 2003), influencing the nutrient availability and its uptake by microalgae (Markou and Georgakakis, 2011).

Temperature affects the protein, lipid, carbohydrate and carotenoid content of algae, but contradictory results have been reported (Markou and Georgakakis, 2011).

Carbon

The carbon content of microalgae ranges between 36 and 65 % (Sydney et al., 2010; Chae et al., 2006). Photosynthetic microalgae use inorganic carbon as carbon source. Since microalgae have a low CO_2-binding capacity of rubisco, and the oxygenase activity of rubisco depends on the CO_2:O_2 ratio, an elevated intracellular CO_2 concentration is a prerequisite (Graham and Wilcox, 2000). Therefore, certain microalgae possess carbon concentrating mechanisms (CCMs) that elevate the CO_2 level at the site of rubisco up to 1000-fold over that of the surrounding medium (Price et al., 2008). Microalgal CCMs involve: (1) cell membrane inorganic carbon transporters/pumps, (2) carbonic anhydrases, (3) specialized cellular structures and (4) calcification (Jansson and Northen, 2010). Whereas CO_2 can directly enter microalgae cells by diffusion, bicarbonate uptake requires transporter systems or prior conversion to CO_2 (Giordano et al., 2005a). Certain cyanobacteria can utilize CO_3^{2-} (Mikhodyuk et al., 2008).

Next to inorganic carbon, certain microalgae can use organic carbon by phagotrophy or osmotrophy. Osmotrophy requires transporters and enzymes to enable assimilation of dissolved organic carbon, such as acetate, glucose, lactate, glutamate, alanine, asparagine and ethanol (Lee et al., 2001). In mixotrophic cultures photo-inhibition is reduced, growth rates are improved and biomass losses during night are lower compared to autotrophic cultures (Markou and Georgakakis, 2011). Certain algae can excrete enzymes, such as protease and lipases, to lyse undissolved organic carbon (Graham and Wilcox, 2000). The organic carbon concentrations in wastewater, measured as COD, range widely from < 30 up to > 150,000 mg COD L^{-1} (Olguín, 2012). Next to the inorganic carbon already present in most wastewaters, microbial conversion of COD produces extra inorganic carbon *in situ***.**

Nitrogen

The N content of microalgae ranges from 1 % to over 10 %, and depends on the amount, availibility and source of N (Markou and Georgakakis, 2011). Certain microalgae show luxury uptake of nitrogen (Becker, 2008). Uptake of NH_4^+, NO_2^-,

NO_3^-, N_2, urea ($CO(NH_2)_2$), NO, NO_2 and organic nitrogen (e.g., amino acids) by microalgae has been shown (Graham and Wilcox, 2000; Nagase et al., 2001; Becker, 2008) (Fig. 1.2). Most algae are able to import NH_4^+ and NO_3^-, but NH_4^+ is often preferred because it can be used in a more direct fashion than NO_3^- to produce N-containing organic compounds (Fig. 1.2.). N-uptake is affected by extracelluar and intracellular enzymes (Fig. 1.2.). Nitrate uptake depends on iron availability because nitrate reductase includes iron, while NH_4^+ uptake does not require iron (Graham and Wilcox, 2000).

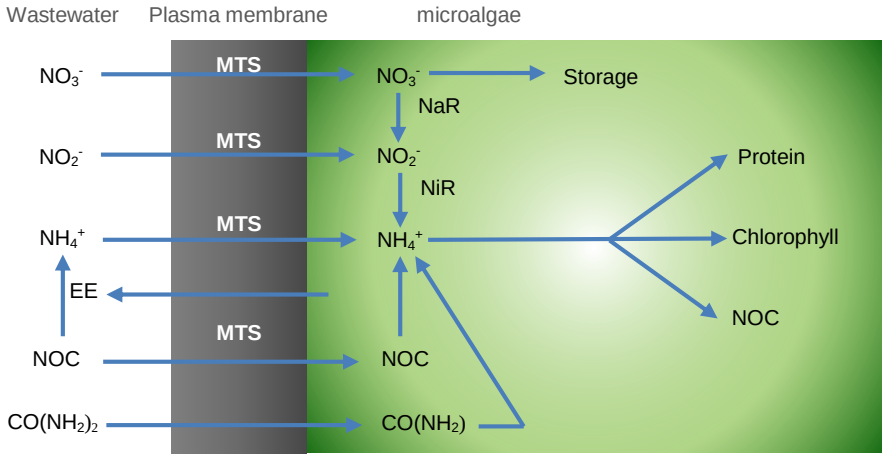


Fig. 1.2. *Overview of all proposed models for nitrogen uptake by microalgae. Following uptake involving membrane transport systems (MTS), ammonium can be converted directly to organic nitrogen and utilized in synthesis of protein, chlorophyll and other N-containing organic compounds (NOC). In contrast, nitrate and nitrite utilization requires additional steps needing nitrate reductase (NaR) and/or nitrite reductase (NiR). Certain NOCs can be directly taken up and lysed in the cell to ammonium or lysed outside the cell by excretion of extracelluar lysing enzymes (EE). Based on Becker, 2008; Nagase et al., 2001; Graham and Wilcox, 2000; Lobban and Harrison, 1994.*

High concentrations of NH_4^+ can inhibit NO_3^- uptake because NH_4^+ represses the synthesis of nitrate reductase, while high NO_3^- concentrations can inhibit NH_4^+ uptake (Markou and Georgakakis, 2011; Mc Carthy, 1981). However, low light conditions can result in a lack of inhibition of NO_3^- uptake by NH_4^+ (Yin et al., 1998). In this way, high turbidity of wastewaters can affect NO_3^- uptake (Cañizares-Villaneuva et al., 1995). In algae reactors with pH values above 9.3, high NH_4^+ concentrations are not stable because free ammonia (NH_3) begins to dominate over ammonium. Free ammonia is toxic to most microalgae (Abeliovich and Azov, 1976)

as it decouples the electron transport in PSII (Muñoz and Guiyesse, 2006). For example, low ammonia concentrations (3.2 mM N-NH_3) were toxic for *Phormidium* sp. (de la Noüe and Bassères, 1989). Increased temperature favours the formation of free ammonia (Markou and Georgakakis, 2011). Also NO_2^- is toxic for certain microalgae (Becker, 2008). Nitrogen fixing cyanobacteria can assimilate N_2 from the atmosphere and reduce it to NH_4^+ using nitrogenase (Graham and Wilcox, 2000). Some microalgae can utilize NO, an intermediate in denitrification, as N source (Richmond, 2004). An increase of the nitrogen load can increase the protein content, while nitrogen limitation can increase the lipid content of microalgal biomass (Markou and Georgakakis, 2011). The TN concentrations in wastewater range widely from < 10 to up to > 2000 mg L^{-1} (Olguín, 2012). High concentrations of nitrogen and the presence of free ammonia and NO_2^- in wastewaters need to be avoided if treatment by microalgae is the aim.

Phosphorous

Microalgae contain around 0.4-3.3 % phosphorous (Shilton et al., 2012). Phosphorous is essential for almost all cellular processes, such as biosynthesis of nucleic acids and phospholipids, and for energy transfer (Becker, 2008). The major form in which microalgae acquire P is as inorganic phosphate (Barsanti and Gualtieri, 2006) (Fig. 1.3.). The phosphate forms in a solution at pH 6-11 are $H_2PO_4^-$, HPO_4^{2-} or PO_4^{3-} (Markou and Georgakakis, 2011). Certain microalgae can produce extracellular phosphatases to hydrolyse organic P prior to its uptake (Becker, 2008). The uptake rate of P is decreased in conditions with non-optimal pH and lack of K^+, Na^+ and Mg^{2+} (Markou and Georgekakis, 2011). Under sufficient P supply, phosphates are accumulated in the algae as acid-labile polyphosphate in large granules, termed luxury uptake in polyphosphate bodies (Graham and Wilcox, 2000). These are metabolized under P deficiency (Becker, 2008). Phosphorous can also be considerably removed by adsorption to the microalgal cell surface mediated by ligand exchange reactions with adsorbed (hydro-)oxides (Sañudo-Wilhelmy et al., 2004). When excess of calcium cations are present, precipitation of positively charged calcium phosphate and its adsorption to negatively charged algal cell walls can occur (Vandamme et al., 2013). This precipitation is function of the calcium concentration, phosphate concentration, pH, temperature, Ca:Mg ratio, carbonate concentration and

extracellular polymeric substance (Larsdotter, 2007; Beuckels et al., 2013). More research is needed to verify whether in wastewater treating algal systems phosphorous is also removed via adsorption to calcite crystals (Karageorgiou et al., 2007) or via indirect adsorption to the microalgal cell surface mediated by ligand exchange reactions with adsorbed cations.

Considering these different microalgal systems for P removal and uptake (Fig. 1.3.), the molar N:P ratio of biomass can largely vary from 3:1 to 100:1 (Craggs et al., 2011; Geider and Laroche, 2002). This differs from the typical microalgal biomass composition of $C_{106}H_{181}O_{45}N_{16}P$ (Park et al., 2011b). Wastewater contains < 1 up to over 600 mg P L^{-1} (Olguín, 2012). An unbalanced N:P ratio in wastewater can lead to P limitation or N limitation of algal growth. The critical N:P ranges that marks the transition from N and P limitation appears to be in the range of 20-50 (Geider and Laroche, 2002). Phosphorous limitation can increase the lipid and carbohydrate content, and decrease the protein and Chl*a* content of microalgae (Richmond, 2004). After phosphorous starvation, the phosphorous uptake rate is much higher than in unstarved algae cultures (Prieto et al., 1997).

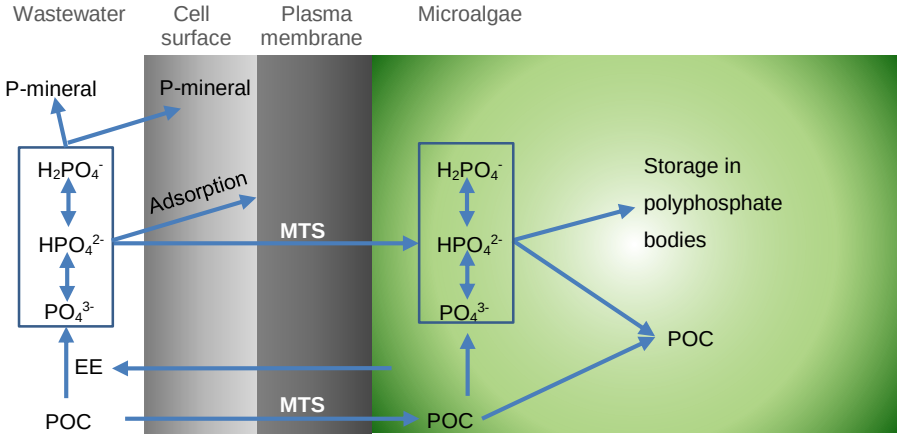


Fig. 1.3. *Overview of all proposed models for P removal by microalgae*
Following uptake involving membrane transport systems (MTS), phosphate can be converted directly to P-containing organic compounds (POC). P can be removed by precipitation into minerals and by adsorption to the cell wall. POC can be uptaken directly or after lysis by extracellular enzymes (EE). Based on Markou and Georgakakis, 2011; Becker, 2008; Barsanti and Gualtari, 2006; Sañudo-Wilhelmy et al., 2004; Graham and Wilcox, 2000.

Other micro- and macronutrients

Next to C, N and P, microalgae commonly require other macronutrients (S, Ca, Mg, K) and micronutrients (B, Br, C, Co, Cu, Fe, Mn, Mo, Ni, Zn) (Graham and Wilcox, 2000). Their presence in wastewater depends on the origin of the wastewater.

Inhibitory substances

The high turbidity and/or dark colour of certain wastewaters can inhibit photosynthetic microalgal growth (Depraetere et al., 2013). Decolorization of piggery wastewater by chitosan (50 mg L^{-1}) and oxidation processes (H_2O_2 and UV) are effective but expensive, whereas decolorization by cationic agents and heavy metals also removes phosphate resulting in P limitation (Depraetere et al., 2013). Moreover, high heavy metal concentrations can inhibit microalgal photosynthesis because they replace or block metal atoms in the active sites of enzymes (Kumar et al., 2010).

pH

Most microalgal species are favored by a pH of 7-8 (Kong et al., 2010), whereas some species are tolerant to a higher pH, e.g., *Spirulina platensis* at pH 9 (Hu et al., 1998), or lower pH, e.g., *Chlorococcum littorale* at pH 4 (Kodama et al., 1993). Increasing the pH by photosynthesis to above 9.5 leads to a shift from NH_4^+ to NH_3. This results in toxicity to the microalgae and loss of N via volatilization (Muñoz and Guieysse, 2006). A pH above 10.5 shifts bicarbonate to carbonate which can only be directly uptaken by certain cyanobacteria, but not by other algae (Graham and Wilcox, 2000). Moreover a high pH leads to phosphate precipitation and affects the heavy metal availability (Park et al., 2011b; Muñoz and Guieysse, 2006).

Cell density

To obtain high biomass productivities, the microalgae density must be set so that exponential microalgal growth is maintained (Becker, 2008). As the microalgal density increases, so does the shading effect algae create and the switch to occurrence of dark respiration (Muñoz and Guieysse, 2006). At high PPFDs, mutual shading can be used to increase the frequency of light/dark cycles at which the cells are exposed to optimize the photosynthetic activity (Richmond, 2004).

Algae grazers and pathogens

Microalgal cultures are susceptible to grazing by herbivorous protozoa, zooplankton (e.g., rotifers) and insect larvae which can reduce microalgae densities to low levels within just a few days (Becker, 2008; van Hamelen and Oonk, 2006; Cauchie et al., 1995). Also parasitic fungi, mainly *Chytridium* and *Aphelidium* sp., can cause unexpected process failures (Becker, 2008). The latter might already be present in the incoming wastewater (Park et al., 2011b). Exudates of microalgal grazers can induce bioflocculation of microalgae (Hessen and van Donk, 1993).

1.3.4. Microalgae and bacteria interactions

The main focus of MaB-S-WWT has been photosynthetic O_2 production and CO_2 uptake by microalgae, and CO_2 production and O_2 uptake by bacteria (Fig. 1.4.). However, microalgae and bacteria do not limit their interactions to a simple CO_2/O_2 exchange (Fig. 1.4.). Given the immense diversity of microalgae and bacteria, the interactions between these organisms are still an open field for further research.

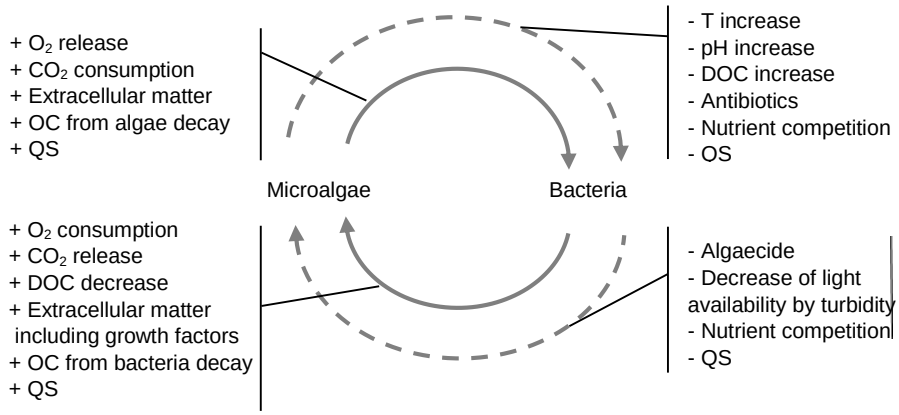


Fig. 1.4. *Positive (plain line) and negative interactions (dashed line) between microalgae and non-photosynthetic bacteria*
OC: organic compound; DOC: dissolved organic compound; QS: quorum sensing i. e. cell-to-cell communication through the release and detection of small signal molecules. Based on Muñoz and Guieysse, 2006; Natrah et al., 2013.

1.3.5. Biotic and abiotic processes involved

Several biotic and abiotic processes involved in solar-based MaB-S-WWT have been reported (Table 1.2.). Wastewater composition, reactor operation, outdoor conditions of light and T, and cell densities can affect these processes and their interplay (Muñoz and Guieysse, 2006; Shilton, 2005). Moreover, other yet to reveal mechanisms may play a role. A tremendous amount of future research is needed if understanding the interplay of all these processes is the aim, because currently only the top of this 'eco-system iceberg' has been barely touched (Dupraz et al., 2009; Shilton, 2005).

Table 1.2. *Overview of reported biotic and abiotic mechanisms affecting the decrease and increase of wastewater parameters in sunlight-powered MaB-S-WWT*
Ma: microalgal process; B: process by non-photosynthetic bacteria; A: abiotic process; VOC: volatile organic compound; C_xH_y: carbohydrate. Based on Dupraz et al., 2009; Muñoz and Guieysse, 2006; Gutzeit, 2006; Shilton, 2005; Richmond, 2004; Hammes, 2003; Graham and Wilcox, 2000; Jenkins et al., 2000; Wolfaardt et al., 1994; Grima et al., 1993.

Parameter	Decrease (removal)	Increase (release)
Inorganic carbon	• Photosynthesis (Ma) • Calcification of cell wall (Ma) • Microbial-influenced precipitation of $CaCO_3$ (Ma, B) • Storage in vacuoles (Ma)	• Heterotrophic processes (B) • Dark respiration (Ma) • Photorespiration (Ma) • Urea uptake (Ma, B) • Release by decay (Ma, B)
Organic carbon	• Settling (A) • Adsorption (Ma, B) • Denitrification (B) • Heterotrophic processes (Ma, B) • Phagotrophy (Ma) • Release of VOC (Ma, B) • Uptake of EPS (Ma, B) • Breakdown by UV-light (A)	• Excretion of EPS (Ma, B) • Decay (Ma, B) • Production of dissolving C_xH_y (Ma, B)
Oxygen	• Photosynthesis (Ma) • Reactor mixing (A) • Denitrification (B)	• Heterotrophic growth (Ma, B) • Nitrification (B) • Dark and photo-respiration (Ma)
Organic N (which is no urea)	• Settling (A) • Ammonification (Ma, B) • Phagotrophy (Ma) • Uptake and assimilation (Ma, B)	• Excretion of EPS (Ma, B) • Decay (Ma, B)
NO_3-	• Assimilation (Ma, B) • Storage in vacuoles (Ma) • Denitrificaton (B)	• Nitrification (B) • Release by decay (Ma, B)
NO_2^-	• Assimilation (Ma, B) • Nitrification (B)	• Nitrification (B) • Release by decay (Ma, B)
NH_4^+	• Anammox process (B; if anoxic)	• Release by decay (Ma, B)

	• Assimilation (Ma, B) • NH_3 stripping at high pH (A, but pH increase is microbial-influenced)	• Ammonification (Ma, B)
$CO(NH_2)_2$	• Assimilation (Ma, B)	• Excretions by predators
N_2	• N_2-fixation (Ma: only cyanobacteria)	• Denitrification (B)
Organic P	• Settling (A) • Uptake and assimilation (Ma, B) • Phagotrophy (Ma) • Conversion by external phosphatase (Ma, B) • Anaerobic conversion to PH_3 gas (B)	• Excretion of EPS (Ma, B) • Decay (Ma, B)
Phosphate	• Assimilation (Ma, B) • Adsorption (Ma) • Luxury-uptake of P (Ma, B) • Precipitation (A, but pH increase is microbial-influenced)	• Dissolvement of precipitates if decreased pH (A) • Release by decay (Ma, B) • Conversion by external phosphatases (Ma, B)
Recalcitrant compound	• Conversion by external enzymes (Ma, B) • Assimilation after lysis (Ma, B) • Enhancement of bacterial degradation by microalgal EPS (Ma)	
Heavy metals	• Assimilation, ion exchange and adsorption (Ma, B) • Chelation to extracellular metabolites (Ma, B) • Precipitation and crystallization on the cell surface (Ma, B) • Precipitation in water (A, but pH increase is microbial-influenced)	• Release by decay (Ma, B)
Pathogens	• Disinfection by UV-light (A) • Excretion antibiotics (Ma) • Disinfection by increased oxygen and increased pH (indirectly by Ma) • Adsorption on cell surface (Ma)	• Pathogen growth (B)
pH	• Heterotrophic processes (A, B) • Nitrification (B) • Photorespiration (Ma) • Uptake of NH_4^+ (Ma, B)	• Photosynthesis (Ma) • Uptake of NO_2^-, NO_3^- (Ma, B) • Denitrification (B) • Uptake of $CO(NH_2)_2$ (Ma, B)

1.3.6. Microalgal bacterial reactor types

Two groups of reactor types for sunlight-powered MaB-S-WWT have been proposed: closed systems, named photobioreactors (PBRs), and open systems. PBRs are often designed as tubular or flat plate reactors arranged in a horizontal, inclined, vertical or spiral manner (Tredici, 1999). In PBRs with wastewater treating microalgal bacterial cultures, problematic biofilm formation is often observed (Arbib et al., 2013) (Fig. 1.5.). This phenomen has been applied in microalgae biofilm PBRs, including tubular reactors with a biofilm on the wall (González et al., 2008) or a biofilm on

bristles in the reactor (Avendaño-Herrera and Riquilme., 2007), and flat plate biofilm reactors, such as the 'algal roof' (Zamalloa et al., 2013), horizontal biofilm flow cells (Boelee et al., 2011) and vertical twin-layer biofilm reactors (Shi et al., 2007).

One of the most applied open MaB-S-WWTs are waste stabilization ponds (WSP). These include a facultative pond with anaerobic, anoxic and aerobic zones, followed by a series of aerobic maturation ponds (Shilton, 2005). In some cases, an algae-free, anaerobic pond is incorporated as a first pond to undertake bulk removal of organic load (Shilton, 2005). Facultative ponds are typically 1.5 m deep. High-rate algal ponds (HRAPs; Fig. 1.5.), originally developed by William Oswald, are shallower (0.2-0.8 m) (Shilton, 2005). In HRAPs paddlewheels, pumps or rotating arms move the water at a velocity around 0.3 m s^{-1} in the raceway-shaped or circular pond (Razzak et al., 2013). In algal turf scrubbers (ATS), wastewater flows over a sloped algae-containing periphyton bed (Pizarro et al., 2006). Also open reactors with periphyton covered rotating disks have been proposed (Pryzytock-Jusiaket et al., 1984).

Fig. 1.5. *Open raceway pond treating sewage (STOWA project, the Netherlands) (left) and biofilm fouling in wastewater treating closed photobioreactor (right)*

Open and closed systems have both advantages and disadvantages (Table 1.3). Biofilm-based reactors might present extra limitations: (1) increased photo-inhibition due to constant exposure to high PPFDs at day, (2) potential risk of clogging due to biomass growth in case of PBRs (Muñoz et al., 2009), and (3) increased sensitivity to temperature fluctuations (Murphy and Berberog, 2012). For wastewater treatment in microalgal bacterial reactors and concomitantly production of low-value biomass, low-cost and easy-to-operate reactor types should be chosen. Therefore, especially in areas where the expenses for land are balanced out by these lower reactor costs, open pond systems seem the best choice for MaB-S-WWT.

Table 1.3. *Comparison of open and closed systems for microalgae systems*
+: advantage; -: disadvantage. Based on Arbib et al., 2013; Park et al., 2011b.

Characteristic	Open system	Closed system
Investment and maintenance costs	+ Low	- High
Construction and operation	+ Easy	- Difficult
Biomass densities	- Low (high if biofilm)	+ High
Photosynthetic efficiencies	- Low	+ High
Risk of predator contamination	- High	+ Low
Biomass productivities	- Low	+ High
Illuminated surface:volume ratio	- Low	+ High
Needed land area	- High	+ Low
Toxic accumulation of O_2	+ Low	- High
Overheating of reactor	+ No problem	- High in summer
Difficult to remove biofouling of reactor wall (if no biofilm reactor)	+ Low	- High

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1.3.7. Nutrient removal rates and efficiencies

Compared to other conventional wastewater treatment systems, information on nutrient removal in MaB-S-WWT is still scarce and limited to a few wastewater types (Table 1.3.). Moreover, outdoor reactor performances of MaB-S-WWT are poorly covered in the scientific literature. The hydraulic retention time (HRT), nutrient removal rates and nutrient removal efficiencies largely vary among wastewater and reactor types (Table 1.4). High removal efficiencies for COD, TN and TP seem possible with moderate HRTs (Table 1.4.).

To maximize photosynthetic aeration and nutrient recovery, both biomass growth and biomass harvesting should be maximized (Shilton et al., 2012). The maximum oxygenation capacities vary strongly amongst the reactor types, with reported values (in g O_2 L^{-1} day^{-1}) of 0.01 in WSP, 0.3-0.4 in HRAP, 5.4-6.9 in a tubular PBR, 6.5-8.3 in a flat plate PBR, 1.8-2.3 in a spiral PBR and 2.4-3.1 in a vertical column PBR (Muñoz and Guieysse, 2006).

Table 1.4. *Overview of performances of sunlight-powered microalgal bacterial reactors treating wastewater*

Wastewater	Reactor	HRT	Influent concentration ($mg\ L^{-1}$)			Removal efficiency (%)			Removal rate ($mg\ L_{reactor}^{-1}\ day^{-1}$)			Biomass productivity ($g\ DM\ m^{-2}\ d^{-1}$)	Ref
		(day)	COD	TN	TP	COD	TN	TP	COD	TN	TP		
Diluted centrate[a]	Biofilm PBR	10	8[b]	67	10[c]	50-100[b]	60-80	54-77[c]	-[d]	4-5	0.6-0.8[c]	0.5	1
Sewage	Biofilm PBR	10	181[b]	91	7[c]	90[b]	70	85[c]	54[b]	6	0.6[c]	2	1
Sewage	Biofilm PBR	5	181[b]	91	7[c]	86[b]	59	57[c]	36[b]	11	0.8[c]	3	1
Sewage	Biofilm PBR	3	181[b]	91	7[c]	86[b]	54	36[c]	60[b]	14	0.8[c]	3	1
Sewage	HRAP	10	~80	~25	~2	-	65	59	-	1.6	0.1	8[e]	2
Sewage	Tubular PBR	5	~80	~25	~2	-	90	87	-	4.5	0.3	22[e]	2
Sewage	Biofilm PBR[f]	1	61	36	1.7	77	54	91	47	19	1.5	2.5	3
Sewage	CSTR+ST[g], I[h]	5-7.5	472	79	11	88	67	44	69	8.8	0.8	9.8	4
Manure effluent	ATS[i]	58-175	-	1600	230	-	51-83[j]	62-91[j]	-	20-80[j]	3-11[j]	2.5-24	5
Carpet industry[k]	Vertical tank	-	-	33-46	6-14	-	-	-	-	-	-	8	6
Carpet industry[k]	Polybag	-	-	33-46	6-14	-	-	-	-	-	-	21	6
Carpet industry[k]	HRAP	-	-	33-46	6-14	-	-	-	-	-	-	6	6
Diluted manure[l]	HRAP	10	500-4400	59-123	-	46-76	48-88	<10	-	-	-	6-28	7
Anaerobic effl.	PBR, I[h]	2	-	43-81[m]	5-10	-	67[m]	98[c]	-	19[m]	7.3[c]	70	8
Anaerobic effl.[n]	HRAP	4-8	273-473[p]	-	-	78-79	-.	-	46-93	-	-	16-21	9

[a] From municipal wastewater treatment plant; [b] TOC; [c] $P\text{-}PO_4^{3-}$; [d] No data; [e] Maximum; [f] Slow sand filter and biofilm algal roof; [g] Continuous stirred tank reactor with settling tank of 300 L; [h] Indoor; [i] Algal turf scrubber; [j] Nutrient recovery in harvested periphyton biomass; [k] Sterilized carpet industry effluent (90 %) + sewage (10 %); [l] 10-20 times diluted manure; [m] $N\text{-}NH_3$; [n] from MWWTP; [p] BOD; [1] Posadas et al., 2013; [2] Arbib et al., 2013; [3] Zamalloa et al., 2013; [4] Gutzeit, 2006; [5] Mulbry et al., 2008; [6] Chinnasamy et al., 2010; [7] de Godos et al., 2009; [8] Ruiz-Martinez et al., 2012; [9] Park and Cragss, 2010.

1.3.8. Challenges in microalgal bacterial treatment of wastewaters

MaB-S-WWTs possess several challenges. Low biomass densities, especially in open ponds systems, lead to large areas needed for wastewater treatment. Therefore decreasing the HRT is important. Second, the C:N:P ratio of several wastewaters differs largely from the optimum for microalgal growth and can lead to nutrient limitation. Third, the effluent might reach a pH above target norms and lead to toxic ammonia concentrations of certain wastewaters, especially during sunny days. Flue gas sparging might enhance both the C:N ratio of wastewater and the reactor pH. Fourth, a strong turbidity and dark colour of certain wastewaters might decrease photosynthetic microalgal growth and oxygenation. Fifth, the poor knowledge of the nutrient removal mechanisms involved in MaB-S-WWT limits the rational optimisation. Sixth, no general conclusions can be made on nutrient removal efficiencies and rates in MaB-S-WWTs, since these depend on various factors, such as the wastewater characteristics, reactor operation (HRT, temperature) and the microalgae and bacteria consortia involved (Olguín, 2012). Therefore, each wastewater type sould be screened. Seventh, there is a need for up-scaling of laboratory experiments by establishing outdoor pilot plants (Olguín, 2012). Last, the separation of the microalgal bacterial biomass from the treated wastewater is crucial in obtaining a biomass-free and dischargeable effluent.

1.3.9. Comparison between conventional activated sludge systems and waste stabilization ponds

Worldwide, the most applied MaB-S-WWTs are WSP systems. However, in Northwestern Europe, their application is limited and most applied systems for treatment of industrial wastewaters and sewage in Northwestern Europe are CAS systems. Therefore, the major features of the latter two systems were compared for sewage treatment to pinpoint their advantages and disadvantages (Table 1.5.; Table 1.6.). A hybrid approach which combines the advantageous bioflocculation and efficient nutrient removal as in CAS systems with the sunlight-powered photosynthetic aeration of WSP systems, is presented in this dissertation: microalgal bacterial floc (MaB-floc) reactors.

Table 1.5. *Comparison of major features of sewage treatment in CAS and WSP*
Based on Gutzeit, 2006; Shilton, 2005; Tchobanoglous, 2003; Mara and Pearson, 1998; Von Sperling, 1996.

Characteristic	**CAS[1]**	**WSP[2]**
Dependence on sunlight	No	Yes
Aeration	Mechanical aeration	Photosynthetic aeration
Energy consumption (kWh pe^{-1} $year^{-1}$)[3]	20-30	~0
Hydraulic retention time (days)	0.4-1.2	12-30
Land requirement (m^2 pe^{-1})	0.2-0.4	1.5-3.5
Reactor depth (m)	2-5	AP: 2-5; FP ~1.5; MP: ~0.5
BOD_5 loading (kg BOD_5 m^{-2} d^{-1})	1.2-1.5[4]	0.010-0.020
Effluent quality: BOD_5, COD, N, P	BOD_5, COD and N below discharge norms; often additional chemical treatment needed to reach discharge norms for P	All below discharge norms if no problematic algal growth in maturation pond; P removal is sunlight dependent
Coliform removal efficiency (%)	65-90	60-99,9
Biomass density in reactor (g TSS L^{-1})	2-6	0.05-0.10
Sludge disposal (m^{-3} pe^{-1} $year^{-1}$)	0.7-1.5	No frequent sludge harvest [5]
Biomass valorisation	Biogas, soil fertilizer	Soil fertilizer
Staff for maintenance and control	High expertise and always presence needed resulting in high costs	Low expertise and no daily presence needed, resulting in lower costs
Capital costs (€ pe^{-1})	30-90	8-23

[1] Conventional activated sludge system including carbon removal and nitrification and not including primary treatment; [2] Waste stabilization pond including anaerobic, facultative and maturation ponds; [3] Population equivalent; [4] In case of 3 m deep tank; [5] Sediment removal once in 1-10 years.

Table 1.6. *Overview of general advantages (+) and disadvantage (-) of CAS and WSP*
Based on Shilton, 2005; Von Sperling, 1996.

CAS	WSP
+ High BOD_5 removal	+ Satisfactory efficiency in BOD_5 removal
+ Possibility of biological N & P removal	+ Efficient pathogen removal
+ Low land requirements	+ Simple construction and operation
+ Operational flexibility	+ Low costs and almost no energy requirements
+ Reliable, provided enough supervision is given	+ Satisfactory resistance to load variations
+ Low possibilities of odours, insects and worms	+ Sludge removal practically not necessary
- High costs	- High land requirements
- High energy consumption	- Possible need to remove algae in effluent to
- Need of sophisticated operation	comply with stringent standards
- High mechanization level	- Performance dependent on climatic conditions
- Relatively sensitive to toxic loads	(temperature and radiation)
- Need to complete sludge treatment and disposal	- Possible insect growth
- Possible problems with noises and aerosols	- Possible odour nuisance in anaerobic pond

1.4. Sunlight-powered microalgal bacterial systems for biomass production

1.4.1. Biomass productivities

High microalgal bacterial biomass productivities may not be the main driver in wastewater treatment systems, as the biomass is a by-product. Nevertheless, biological wastewater treatment is intrinsically linked to biomass production and it can be assumed that its valorisation is of major importance for the feasibility of this technology. Wastewater treating HRAPs produce 0.5-28 g dry matter (DM) m^{-2} day^{-1} (Table 1.5). This is similar or lower as compared to monoculture HRAPs producing 14-40 g DM m^{-2} d^{-1} (Park et al., 2011b). Based on a maximum photosynthetic efficiency in HRAPs of 1.3-2.4 % of the solar energy, an average solar radiation of 23.5 MJ and an algae energy content of 21 kJ g^{-1}, a maximum productivity of photosynthetic biomass of 27 g DM m^{-2} day^{-1} can be obtained in temperate climates (Park et al., 2011b). Additional to photosynthetic biomass growth, heterotrophic biomass growth and addition of suspended solids present in the wastewater are expected to increase the biomass productivities in microalgal systems for wastewaster treatment. However, literature on this is scarce.

Reactor operation factors which affect microalgal growth include mixing, HRT, reactor depth, illuminated surface:reactor volume, harvest frequency, biomass density, T and pH control, and operation modes, e.g., continuous stirred tank reactor with settling tank (CSTR-ST) or sequencing batch reactor (SBR) (Richmond, 2004).

Dominant microalgal species in MaB-S-WWT are specific for each study and include *Actinastrum* sp., *Micractinium* sp., *Pediastrum* sp., *Coelastrum* sp., *Chlorella* sp., *Ankistrodesmus* sp., *Scenedesmus* sp., *Phormidium* sp. (references of Table 1.5.). Microalgal predators can cause a serious decrease in biomass productivities. A daily short period (1 h) of low O_2 levels can suppress the growth of higher aerobic organisms (Richmond, 2004). As many zooplankton species can survive extended periods of low O_2 levels (Schluter and Groeneweg, 1981), pH adjustment up to 11 can offer an alternative method of control of these species (Beneman et al., 1978). To control grazer populations of chironomid larvae in algal turf scrubbers, the addition of *Bacillus thuringiensis* has been applied (Mulbry et al., 2008).

1.4.2. Biomass harvesting

An effective separation of biomass from the treated wastewater is needed to prevent washout of biomass during semi-continuous operation and to enable discharge of biomass-free effluent and high recovery of biomass. The main difficulties in microalgae removal from the treated wastewater arise from the relatively low biomass densities of 0.3-5 g L^{-1} (Brennan and Owende, 2010) and microalgal characteristics. Suspended microalgae are small (often < 20 µm; Park et al., 2011a), have a similar density to water (1.08-1.13 g mL^{-1}) (Lavoie and de la Noue, 1987) and have a negative charge of the cell surface particularly during exponentional growth (Moraine et al., 1979). Microalgal biomass harvesting, i.e. biomass removal from the reactor, involves two steps: (1) concentrating of the reactor liquor to a slurry of 2-10 % DM and (2) dewatering of the biomass to a cake of 15-40 % DM (Uduman et al., 2010). Several harvesting technologies are available (Table 1.7.).

Table 1.7. *Overview of technologies for microalgal biomass harvesting*

Technology	**Resulting solid content (% DM)**	**Electricity consumption**		**Limitations**	**Ref.**
		(kWh kg^{-1} DM)	**(kWh m^{-3})**		
Upconcentrating					
DAF[a]	1-10	0.07-1.25	10-20	Electrode replacement	1, 2
Lamella separ.[b]	0.5-1.5	-[f]	0.1	Poor reliability, slow	1
Natural filtration	1-6	-	0.4	Periodic filter replacement	1
Electrocoagulat.[c]	3-5	-	0.8-1.5	Electrode replacement	1
Autoflocculation	-	0	0	Effluent pH above target norm	1
Coagulation-flocculation	<22	-	-	Expensive flocculants, contamination issues	1
Dewatering					
Centrifuging	5-30	0.027-0.95	8	High investment & energy cost	1, 2
Vacuum filter	3-37	5.9	-	Slow, high energy	2, 3
Belt press	10-90	0.4-0.7	-	High capital cost	2
Cham. filt. Press[d]	5-27	-	0.88	Periodic replacement screens	1
MF/UF[e]	10	0.05-0.5	-	Fouling	2
Solar drying bed	4-40	0.25-0.5	-	Altered texture and color	2, 3

[a] Dissolved air flotation; [b] Gravity sedimentation with lamella separator; [c] Electrocoagulation; [d] Microfiltration/ultrafiltration with a polymer membrane; [e] Chamber filter press; [f] No data; [1] Uduman et al., 2010 and references therein; [2] Udom et al., 2013 and reference therein; [3] Show et al., 2013 and references therein.

Information on the cost for algae harvesting from MaB-S-WWT is scarce. In microalgae monoculture systems, biomass harvesting can account for 20-60 % of the total cost of microalgae production (Grima et al., 2003; Olguín, 2003). Disproportional harvesting costs can jeopardize the economic viability of MaB-S-WWT (Olguín, 2003). In (semi-)continuous MaB-S-WWT it is important that effluent can be discharged without the need for an expensive biomass harvesting technique. Imbedment of microalgae in alginate or carrageenan gel matrices circumvents this harvesting issue, but these matrices are costly, degrade easily (Su et al., 2009) and show decreased growth rates in the matrix center (Medina, 2006). A promising but underexplored strategy is bioflocculation of microalgae in the reactor (Gutzeit, 2006). Microalgal flocs settle by gravity enabling effluent discharge without any costs for biomass separation. Moreover, this gravity settling provides a free upconcentrating step during harvesting, similar as in activated sludge systems.

1.4.3. Biomass valorisation

Wastewater fed microalgae have been used for lab-scale production of oil (Kong et al., 2010), biodiesel (Olguín, 2012), biogas (Zamalloa et al., 2011; De Schampheleire and Verstraete, 2009), bio-ethanol (Grima et al., 2003), fertilizer (Muñoz and Guieysse, 2006), animal feed ingredients (Natrah et al., 2013) and high-value molecules such as pigments, amino acids and poly-β-hydroxybutyrate (PHB) (van der Ha et al., 2012; Cardozo et al., 2007). These biomass valorisation pathways are technically feasible, but in most cases their economical feasibility on industrial scale remains to be demonstrated. Moreover, consumer perception and legislation issues often confine additional restrictions, especially for feed applications. Other options of interest for low-value valorisation of microalgal biomass include pyrolysis and biochar, hydrogen production, gasification and direct combustion (Rawat, 2011). Algal metabolites with economic impact are: sterols, phycocolloids, lectins, pigments, amino acids, halogenated products, polyketides and toxins (Cardozo et al., 2007), insecticides and anti-fouling agents (Ducat et al., 2011). Options for economic retrieval of these compounds from wastewater fed microalgal biomass are yet to be shown.

Biomass obtained during treatment of wastewater which is not disinfected is often a mix of various microalgae and bacterial species. For example, the ratio of

algae:bacteria cell numbers in HRAPs treating sewage was around 1:100 (Oron et al., 1979). Natrah et al. (2013) pointed out that a combination of microalgae and bacteria give often better results as aquaculture feed than using them separately. In the so-called greenwater techniques in aquaculture, next to microalgae also bacteria are present. The bacteria levels in these greenwater techniques can reach levels up to 7-fold higher than that of conventional clear water systems (Salvensen et al., 1999). In this regard, aquaculture might be a niche opportunity for microalgal bacterial biomass valorisation. Especially the antibiotic activity of microalgal bacterial biomass is of interest in aquaculture because of the health risks associated with the use of conventional antibiotics (Natrah et al., 2013). Microalgae can provide a vector for bacteria with antibiotic activity (Avendaño-Herrera and Riquelme, 1999), produce antibiotics (Ohta et al., 1995) and/or interfere in cell-to-cell communication of pathogenic bacteria (Natrah et al., 2012). Other microalgal compounds of interest for aquaculture are essential amino acids, polyunsaturated fatty acids (PUFA) such as eicosapentaoenic acid (EPA) and docosahexaenoic acid (EPA), and pigments such as carotenoids, β-carotene and astaxanthin (Cardozo et al., 2007). Crustaceans are unable to synthetize carotenoids *de novo* and require astaxanthin (or appropriate precursors) in their diet to acquire the adequate color for food market acceptance (Cardozo et al., 2007). In salmon production, astaxanthin can account for more than 15 % of the total production costs (Mann et al., 2000). Moreover, these pigments are also UV-light protectors, immunity enhancers, a source of pro-vitamin A and inflammation suppressors (Brennan and Owende, 2010).

1.4.4. Challenges in biomass production and valorisation

Wastewater fed microalgal bacterial biomass harvesting and valorisation is still in its infancy. Cost-effective harvesting and a high value valorisation pathway are a well-hidden holy grale for a cost-effective MaB-S-WWT. Large potential lies in bioflocculation of microalgal bacterial biomass. The combined presence of a consortium of microalgae and bacteria may provide an added value in niche applications such as for aquaculture feed production. As outdoor conditions alter the biomass productivity, composition and community structure, outdoor pilot reactors are needed to confirm proof-of-principle lab-scale studies.

1.5. Bioflocculation of microalgae and bacteria

1.5.1. Bioflocculation

The term bioflocculation of microalgae has been used in literature to describe microalgal floc formation obtained via several strategies:

- Flocculation of one microalgal species and a consortium of bacteria originating from activated sludge, termed 'symbiotic algal bacterial floc' (Su, 2012b; Weinberger, 2012; Gutzeit, 2006; Medina, 2006);
- Flocculation of several microalgal species and bacterial species, termed 'Activated algae' (Doran and Boyle, 1978) or microalgal bacterial flocs (MaB-flocs) (this dissertation);
- Self-aggregation of several cells of one microalgal species, also termed colonial microalgae, such as *Scenedesmus* sp. (Graham and Wilcox, 2000), *Pediastrum* sp. (Park et al., 2011b) and *Phormidium* sp. (Olguín, 2003);
- Flocculation of microalgae sp. with fungi (Zhang and Hu, 2012);
- Flocculation by adding one bioflocculant-producing bacterial species to the produced microalgae during harvesting (Lee et al., 2009);
- Flocculation of a non-flocculating microalga species with a second self-aggregating microalgal species (Salim et al., 2012);
- Flocculation of microalgae by bioflocculants without the presence of the bioflocculant producing organisms; examples are exudates of *Daphnia* sp. (Hessen and van Donk, 1993) and of various bacteria (Oh et al., 2001).

In the first three strategies, the aim is to grow the produced microalgal flocs in reactors. In the other strategies microalgal growth and flocculation are separated in two steps: (1) microalgae cultivation in suspension until harvest and (2) flocculation of microalgae by the addition of other microorganisms or bioflocculant.

Bioflocculation should not be confused with autoflocculation, however in outdoor reactors with high pH it is difficult to distinguish these two phenomens. Autoflocculation or co-precipitaton of microalgae is the spontaneous aggregation of particles associated with the increased reactor pH above 10 as a result of the photosynthetic process (Sukenik and Shelef, 1984; Nurdogan and Oswald, 1995). During this process, algae are entrapped in the calcium and phosphorous precipitates.

1.5.2. Factors influencing bioflocculation

In general, flocculation of suspensions of particles, such as microalgae, can be attributed to five common mechanisms: (1) charge neutralization (negative surface charge is cancelled by positively charged ions or polymers), (2) electrostatic patch mechanism (charged polymer binds to a particle of opposite charge), (3) bridging (polymers or charged colloids binds to the surface of two particles), (4) sweeping flocculation (particles are entrapped in a massive precipitation of minerals) (Vandamme et al., 2013). In microbial flocculation, the displacement of the water film by hydrophobic groups and electrostatic forces are of importance (Medina, 2006). Information on which of these mechanisms plays a role in bioflocculation of microalgae is scarce.

Formation of microalgal (bacterial) flocs is influenced by extracellular polymeric substances (EPS), presence of bacteria, hydrophobicity of cells and EPS, charge of the cell surface and presence of cations functioning as molecular bridges. In general, microalgae increase their EPS production at the end of their growth phase which increases floc formation (Lavoie and de la Noue, 1987). Also light and temperature have an effect on microalgal EPS production (Wolfstein and Stal, 2002). Certain microalgae, such as *Phormidium* sp., excrete bioflocculants (Bar-or and Shilo, 1987). Microalgae flocculation is enhanced by bacterial EPS and presence of bacteria (Lee et al., 2013; Kim et al., 2011; Grossart et al., 2006).

The hydrophobicity and charge of the cell surface depend on the microbial species, growth phase, pH and nutrient availability (Medina, 2006). During exponential growth, algal cells carry a negative charge on the cell surface preventing cell aggregation (Becker, 2008). The addition of cationic metal ions such as Ca^{2+} and Fe^{3+} can neutralize this charge. This leads to cell aggregation (Lee et al., 2013). In activated sludge flocs, the ratio of monovalent ions:bivalent ions should be lower than 2 to avoid deteriation of sludge due to displacement of multivalent by monovalent ions (Higgens and Novak, 1997). Medina (2006) found that during formation of symbiotic microalgal bacterial flocs electrostatic forces were a key factor, while molecular bridging interactions were dominant in older flocs. The effects of all the interactions between algae and bacteria, as summarized in Section 1.3.4., on floc formation remain to be proven.

1.5.3. Floc structure

The floc structure affects floc settling. Calcium concentration of the biomass, hydrophobicity and filament index have a significant effect on the settleability of the symbiotic algal bacterial flocs (Medina, 2006). Filamentous microorganisms are considered to confer a backbone structure to the floc and to improve the floc strength (Jenkins et al., 2004). Nevertheless, excessive presence of some filamentous species, e.g., *Thiotrix* sp., results in foam formation and bulking sludge (Jenkins et al., 2004).

The floc structure is determined by operational process parameters such as the influent composition, HRT, sludge retention time (SRT) and predators (Medina, 2006). Under starvation conditions, bacteria become more hydrophobic which can favor aggregation (Bossier and Verstraete, 1996). A decrease in nutrient loading of MaB-S-WWT results in an increased presence of microalgae in the biomass, improved settling and decreased concentrations of suspended algae in the effluent (Medina, 2006). In activated sludge, an increased SRT, and thus an increased cell age, favors the formation of stronger flocs (Tchobanoglous, 2003). Also the presence of predators is considered as beneficial for floc formation (Jenkins et al., 2004). However, it is not clear if agglomeration corresponds to a response to predator presence (Bossier and Verstraete, 1996).

1.5.4. Reactor start-up and operation

Attemps to grow introduced microalgal species in wastewater fed HRAP as monocultures for periods greater than 3 months have all failed due to contamination by other native algae and/or zooplankton (Park et al., 2011b). Therefore, microalgal systems for wastewater treatment should preferably start from an inoculum containing a consortium of microalgal species. In this regard, during reactor operation the most suitable species will be selected, i.e. highest growth rates and tolerance to seasonal and diurnal variations in outdoor conditions.

One of the major advantages of microalgal bacterial flocs is that these flocs settle by gravity enabling discharge of biomass-free effluent without the need for expensive harvesting technologies. In this way, microalgal bacterial floc reactors for wastewater treatment can be operated in continuous stirred reactors with settling tank (Weinberger et al., 2012; Gutzeit, 2006; Medina, 2006) or sequencing batch reactors (SBR; this study).

1.5.5. Challenges in bioflocculation

All the pieces of the complex puzzle of mechanisms behind microalgal bioflocculation and the effect of wastewater composition, still need to be reveiled. Especially the organic carbon:inorganic carbon ratio (OC:IC) in wastewater might be of importance, since these strongly influence the presence of OC consuming heterotrophic and IC consuming autotrophic species. Anno 2008 (at the start of this dissertation), published reports on microalgal bacterial floc reactors were restricted to treatment of sewage. Since each wastewater type is unique in composition, experimental work needs to be performed to screen the potential of microalgal floc reactors for various wastewaters. Since calcium ions have shown to play an important role in bioflocculation, a niche opportunity of microalgal bacterial floc reactors might be treatment of calcium-rich wastewaters. Besides, calcium removal by MaB-flocs can be interesting for the industry, especially if cycling of treated wastewater is the goal.

The dominance of specific microalgal species in microalgal bacterial flocs can be advantageous for biomass valorisation. However, microalgal species control has yet to be achieved in wastewater treatment ponds and algal dominance and species interactions are still poorly understood (Park et al., 2011b). Nevertheless, the idea that polycultures are better than monocultures is gaining acceptance in agriculture and forestry, and is starting to appear in studies on microalgal systems, especially for wastewater treatment (de-Bashan and Bashan, 2010). Also in nature, most microalgae are found in association with other aerobic microorganisms (Mouget et al., 1995). Moreover, most raw industrial wastewaters contain bacteria, making it difficult to grow monocultures of axenic microalgae in wastewater. In this dissertation, **m**icro**a**lgal **b**acterial flocs or **MaB**-flocs are developed with a consortium of several microalgal species and bacterial species.

1.6. Objectives and outline of this research

Sunlight-powered microalgal bacterial systems for wastewater treatment (MaB-S-WWT) are based on photosynthetic aeration which replaces the costly mechanical aeration of conventional activated sludge systems. Moreover, microalgae scavenge nutrients from the wastewater, in line with the requested paradigm shift towards nutrient-recovering wastewater treatment plants. However, the amount of publications on these microalgal systems for wastewater treatment largely outnumbers the amount of their industrial application, especially in Northwest Europe. As reviewed in **Chapter 1**, several challenges hamper their implementation. This dissertation aims at addressing some of these challenges.

A major hurdle in MaB-S-WWT is the separation of the microalgal bacterial biomass from the treated wastewater. Therefore, in this dissertation a novel concept is presented: microalgal bacterial flocs in sequencing batch reactors (**MaB-floc SBRs**) for wastewater treatment based on bioflocculaton of a consortium of local autotrophic and heterotrophic species (Fig. 1.6).

Since wastewaters largely differ in their inorganic carbon:organic carbon ratio, the effect of this **IC:OC ratio** on wastewater treatment and MaB-floc characteristics was investigated (**Chapter 2**).

MaB-floc treatment of primary treated sewage entails several challenges. The C:N ratio is too low to scavenge all N from sewage. Moreover, photosynthesis increases the reactor pH to values above the discharge norm and microbial optimum, and can lead to nitrogen loss via ammonia volatilization. Flue gas supplementation may provide a solution to all these challenges. However, the flue gas flow rate has to be limited to avoid algal toxicity and to reach stringent off gas and effluent limits. Therefore, the potential of MaB-floc SBRs was investigated for the secondary treatment of **sewage sparged with different flue gas flow rates** (**Chapter 3**).

Next to CO_2, **flue gas** contains several other **compounds** which can positively or negatively interact with microalgae. To better steer and engineer flue gas-fed microalgae reactors, all these compounds need to be considered. Therefore, the chemical composition of flue gas, current flue gas treatment technologies, and biochemical interactions of flue gas compounds and microalgae were reviewed. Based on this review, innovative biotechnological opportunities are envisaged (**Chapter 4**).

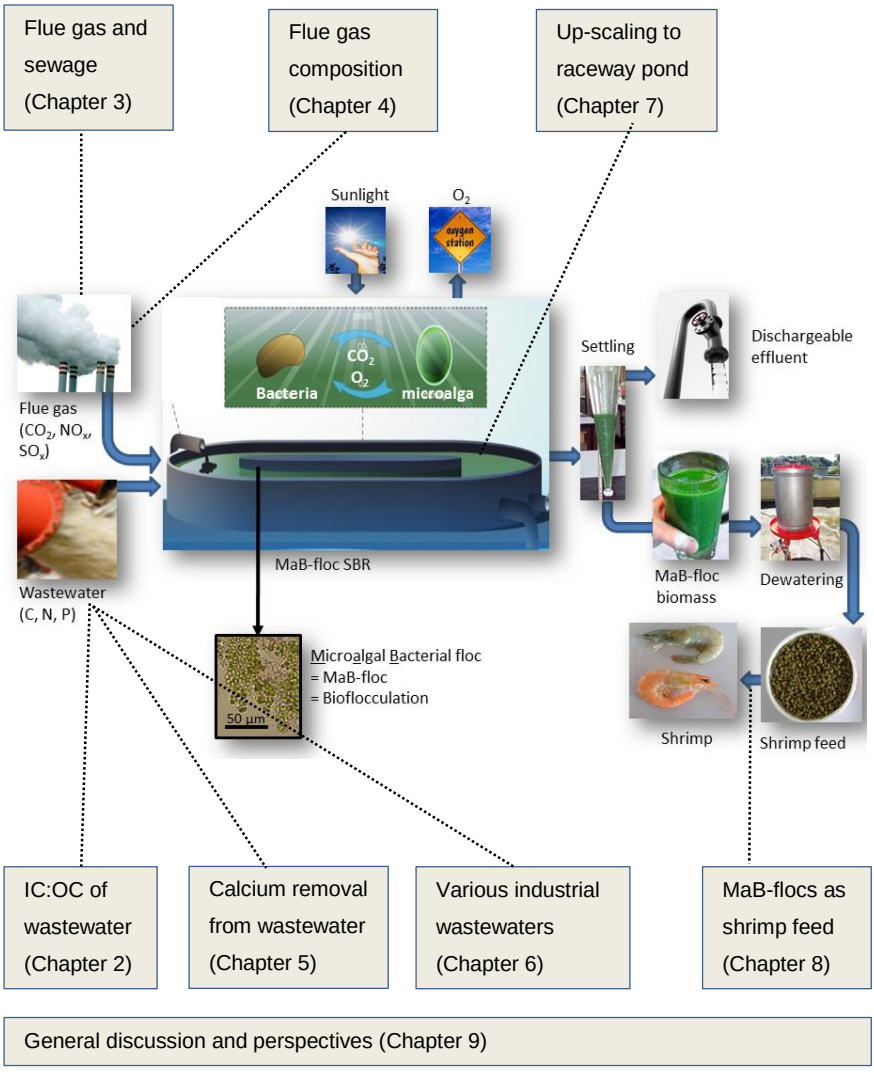


Fig. 1.6. Overview of all chapters of this work on MaB-floc SBR technology

To find a niche opportunity for MaB-floc SBRs, it was investigated whether MaB-floc SBRs significantly **remove calcium** from calcium-rich paper mill UASB effluent (**Chapter 5**).

Since the composition of (agro-)industrial wastewaters largely varies, research efforts should be undertaken to effectively pinpoint its valorisation potential and assess its industrial applicability. Therefore, the potential of MaB-floc SBRs was

screened for **wastewaters from various (agro-)industries**: aquaculture, manure treatment, food-processing industry and chemical industry (**Chapter 6**).

Indoor lab-scale reactor conditions strongly differ from outdoor reactor conditions. Indoor reactor performances can not be simply extrapolated to outdoor reactor performances. To screen the potential of MaB-floc SBRs for outdoor application in Northwest Europe, aquaculture wastewater was treated in an **outdoor MaB-floc raceway pond with flue gas injection**. Moreover, the effect of up-scaling from indoor reactors to outdoor pond was evaluated on experimental data (**Chapter 7**).

MaB-flocs reactors not only treat wastewater, but also produce microalgal bacterial biomass. This biomass needs valorisation. To screen whether the outdoor MaB-floc **biomass** can be **valorised as aquaculture feed**, MaB-flocs were added to the diets of Pacific white shrimp (*Litopenaeus vannamei* Boone, 1931). The effects on the growth of shrimp and of the colour of cooked shrimp were investigated (**Chapter 8).**

Chapter 9 finishes with a **general discussion** and **future perspectives** based on the obtained results.

Observe and interact -

Beauty is in the eye of the beholder.

(Holmgren, 2005)

Picture on previous page:
Periphyton growing in a settling tank containing secondary sewage effluent (Aquafin, Harelbeke, Belgium).

CHAPTER 2

Microalgal bacterial floc properties are improved by a balanced inorganic:organic carbon ratio

Abstract

Microalgal bacterial floc (MaB-floc) reactors have been suggested as a more sustainable secondary wastewater treatment. In this study, it was investigated whether MaB-flocs could be used as tertiary treatment. Tertiary influent has a high inorganic carbon:organic carbon ratio, depending on the efficiency of the secondary treatment. In this study, the effect of this inorganic:organic carbon ratio on the MaB-flocs performance was determined, using three sequencing batch reactors. The MaB-flocs were fed with synthetic wastewater containing 84, 42 and 0 mg C-$KHCO_3$ L^{-1} supplemented with 0, 42, 84 mg C-sucrose L^{-1}, respectively, representing inorganic versus organic carbon. Bicarbonate significantly decreased the autotrophic index of the MaB-flocs and resulted in poorly settling flocs. Moreover, sole bicarbonate addition led to a high pH of 9.5 and significant lower nitrogen removal efficiencies. Sucrose without bicarbonate resulted in good settling MaB-flocs, high nitrogen removal efficiencies and neutral pH levels. Despite the lower chlorophyll *a* content of the biomass and the lower *in situ* oxygen concentration, 92-96 % of the soluble COD-sucrose was removed. This study shows that the inorganic:organic carbon ratio of the wastewater is of major importance and that organic carbon is requisite to guarantee a good performance of the MaB-flocs for wastewater treatment.

Chapter redrafted after:

Van Den Hende S., Vervaeren, H., Saveyn, H., Maes, G., Boon, N., 2011. Microalgal bacterial floc properties are improved by a balanced inorganic/organic carbon ratio. Biotechnol. Bioeng. 108, 549-558.

2.1. Introduction

Today, environmental biotechnology is getting a powerful pull from one of the most pressing needs of human society: sustainability (Rittmann, 2006). Microalgae, having exerted profound effects on our planet and its biota for billions of years, may play an important role in the development of sustainable environmental technology. They have showed great potential for wastewater treatment and carbon dioxide mitigation (Soletto et al., 2008; Wang et al., 2008; Doucha et al., 2005), while producing novel biomass with higher yield potentials (up to 50 ton dry matter ha^{-1} $year^{-1}$ in Northwest Europe; Wijffels, 2008) and lower water demand than terrestrial biomass (Gouveai and Oliveira, 2009; Posten and Schaub, 2009). Microalgal biomass applications are numerous (Loutseti et al., 2009; Spolaore et al., 2006). Microalgae can contribute in the development of more integrated and cost-efficient environmental technologies (Avagyan, 2008; Rittman, 2008; Gronlund et al., 2004) such as wastewater treatment.

Conventional wastewater treatment with activated sludge has a large carbon footprint due to the high energy use for aeration and CO_2 emission from organic carbon conversion by heterotrophic bacteria (Cakira and Stenstromb, 2005). By using microalgal bacterial consortia for wastewater treatment, CO_2 can be converted to biomass while O_2 is produced *in situ*. This O_2 is utilized by heterotrophic bacteria for the aerobic biodegradation of organic compounds to CO_2, which in turn is assimilated by the microalgae. Consequently, photosynthetic CO_2 uptake increases the overall biomass yield, whereby the concomitant generated biomass furnishes an interesting raw material (Mulbry et al., 2005; Muñoz et al., 2005; Sandau et al., 1996). The interaction between microalgae and bacteria in wastewater treatment has been studied over 30 years ago (Muzafarov and Taubaey, 1983). However, challenges lie within the separation of the microalgal bacterial biomass (Richmond, 2004). The use of microalgal bacterial flocs has the advantage of a simple separation of the biomass from the liquid by settling (Medina and Neis, 2007; Gutzeit et al., 2005). More research is needed to understand and control the mechanisms of bioflocculation (Muñoz and Guieysse, 2006).

Despite this wide range of advantages, the intrinsic land requirement has hindered the widespread implementation of this sunlight driven technology for the treatment of primary effluent (Bordel et al., 2009). This technology could be a

promising alternative for the polishing of secondary effluent, requiring less land area. The ratio of inorganic carbon:organic carbon (IC:OC) of secondary effluent depends on the treatment efficiency, but this ratio is in general high in secondary effluent (Ayaz, 2008; Tassoula et al., 2007). It is expected that a certain amount of organic carbon is needed to obtain good microalgal bacterial floc characteristics, since the exchange of O_2 and CO_2 between microalgae and bacteria stimulates the formation of microalgal bacterial flocs (Medina and Neis, 2007; Gutzeit et al., 2005).

In this study, the effect of the IC:OC ratio on the MaB-floc reactor performance (nutrient removal, effluent turbidity and floc settleability) was determined. Therefore, lab-scale experiments were performed in three parallel, sequencing batch reactors (SBRs) where MaB-flocs were fed with wastewater containing different levels of OC and IC. The turbidity of the effluent, pH, nitrogen and chemical oxygen demand (COD) removal were followed up. Bicarbonate was used as the IC source, because mainly present in this form in secondary wastewater effluent (Fang et al., 2001). Sucrose was used as organic carbon source. To exclude a secondary effect of the nitrogen source, nitrate was used as nitrogen source in all reactors. Furthermore, it was tested whether the autotrophic index (AI) and settleability of the flocs shifted by applying an inorganic carbon source.

2.2. Materials and methods

2.2.1. Preparation of biomass, culture conditions and microorganisms

The biomass was prepared using an algae culture medium based on 3N-BBM+V medium (Schlösser, 1997), further in the text indicated as synthetic wastewater, containing (mg L^{-1} deionized water): $NaNO_3$, 121.00; $CaCl_2.2H_2O$, 4.00; $MgSO_4.H_20$, 4.00; $K_2HPO_4.3H_2O$, 4.05; KH_2PO_4, 9.80; NaCl, 4.00; Vitamin B1, 0.12; Vitamin B12, 0.0012 and trace element solution, 0.24 ml L^{-1}. Trace element solution consisted of (mg L^{-1}) Na_2EDTA, 750.0; $FeCl_3.6H_2O$, 970.0; $MnCl_2.4H_2O$, 41.0; $ZnCl_2.6\ H_2O$, 5.0; $CoCl_2.6\ H_2O$, 2.0; $Na_2MoO_4.2H_2O$, 4.0. The reactors were fed with culture medium which had been stored at 7 °C. All reactors were inoculated with 1 L of an undefined consortium of freshwater microalgae (0.50 g VSS L^{-1} in experimental run I; 0.25 g VSS L^{-1} in run II). The reactors were supplemented with 0.2 L activated sludge liquor (4.5 and 4.7 g VSS L^{-1} respectively) collected from the aeration basin of a municipal wastewater treatment plant (Aquafin, Harelbeke, Belgium), and with 0.3

L synthetic wastewater. In experimental run I, the microalgae were collected in spring 2009 from a lab-scale photobioreactor (Enbichem, Kortrijk, Belgium), while in experimental run II microalgae were collected in summer 2009 from a local natural pond nearby a coal power plant (Electrabel, Ruien, Belgium). The microalgae consortium was a non-defined culture of microalgal species containing *Chlorella* sp., *Pediastrum* sp. and *Scenedesmus* sp. To obtain good settling microalgal bacterial flocs, the microalgal bacterial biomass was operated 3 weeks in 3 parallel SBRs (as described in Section 2.2.2.) with synthetic wastewater prior to starting the experimental run (data not shown).

2.2.2. SBR set-up and operation

Experiments were performed in 3 parallel run SBRs exposed to a light: dark illumination cycle of 15:9 h to mimic a 24 h day:night cycle. The photosynthetic active photon flux density (PPFD) at the water surface level was 100 µmol PAR photons $m^{-2} s^{-1}$ (Field Scout Quantum Light Meter, USA), provided by 6 yellow and 6 cool white fluorescent lamps (36W/840, Philips, The Netherlands). The reactors consisted of Erlenmeyer flasks of 2 L with an effective volume of 1.5 L. The reactors were operated in 2 cycles of 5 hours and one cycle of 14 hours per day (Table 2.1.). To favour microalgal growth and to avoid a low oxygen level when feeding, the reactors were only fed during illumination. To avoid denitrification, the dark cycle started 3 hours after feeding. Only in the reaction phases, the reactors were magnetically stirred (500 rpm; Heidolph, UK). Throughout the study, the SBRs were operated at 23-29 °C. Feeding and decanting were done by peristaltic pumps (Watson & Watson, USA). All feeding, mixing and decanting functions were controlled with programmed timers. The SBRs were inoculated with 1.0 g L^{-1} TSS microalgal bacterial biomass.

The experiment was performed two times (experimental run I and II). The reactors (labelled RB-I, RBS-I and RS-I in run I; RB-II, RBS-II and RS-II in run II) were fed with synthetic wastewater containing bicarbonate (B) and/or sucrose (S; 224 mg COD L^{-1}), representing inorganic and organic carbon, respectively (Table 2.2). The average hydraulic retention time (HRT) was 0.67 days. Furthermore no additional aeration was performed, therefore restricting oxygen supply to in situ biological oxygen production. Nitrogen was supplied as nitrate, as the influent simulates a secondary effluent from a wastewater treatment plant.

Table 2.1. *Operation of the MaB-floc SBRs: 2 cycli of 300 min followed by a third cyclus of 840 min per day*

Cyclus	Phase	Duration (min)	Illumination[a]	Stirring[b]
1, 2	Influent addition	15	L	-
	Reaction	225	L	S
	Settling	30	L	-
	Effluent withdrawal	30	L	-
3	Influent addition	15	L	-
	Reaction	165	L	S
	Reaction	540	-	S
	Reaction	60	L	S
	Settling	30	L	-
	Effluent withdrawal	30	L	-

[a] L: reactors were illuminated with 100 µmol PAR photons m^{-2} s^{-1}; -: reactors were operated in dark; [b] S: reactors were stirred; - : reactors were not stirred.

The reactor cultures were sampled by collecting 150 mL liquor twice a week 1.5 hour before the settling phase of the second SBR cycle. These samples were analyzed for chlorophyll *a (*Chl*a)*, total suspended solids (TSS) and volatile suspended solids (VSS). Dissolved oxygen (DO) was at least once a week measured in the reactors one hour before the settling period of the second SBR cycle. Subsequently, before settling in the reactors started, 1 liter of reactor liquor was temporarily (30 minutes) redrawn from the reactors to measure the sludge volume index (SVI). Every 2 weeks, samples were taken to measure the floc size distribution. Microscopic observations were performed once a week. Twice a week, the effluent of the first cycle was analyzed for turbidity, pH, soluble chemical oxygen demand (SCOD), $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$. Both experimental runs lasted minimum 24 days.

To verify whether the differences in floc characteristics were due to a higher pH and/or to the presence of bicarbonate, a third experiment was set up (Table 2.2). On day 33 of experimental run II, the MaB-flocs of RS-II were transferred into 2 SBRs, resulting in a third experimental run. Both reactors (labelled RBSP-III and RS-III) were operated identically, except for the carbon source and concentrations and pH of the influent (Table 2.2.). The influent pH of RBSP-III was adjusted to 8.5 with a solution of 0.1 M NaOH.

Table 2.2. *Overview of the concentration of inorganic and organic carbon of the influent of 8 MaB-floc SBRs during 3 experimental runs*

Run	Reactor	Period (days)	C-Sucrose (mg C L^{-1})	C-$KHCO_3$ (mg C L^{-1})
I	RB-I	1-30	0	84
I	RBS-I	1-30	42	42
I	RS-I	1-30	84	0
II	RB-II	1-28	0	84
II	RBS-II	1-28	42	42
II	RS-II	1-28	84	0
III	RBSP-III	1-10 [a]	84	0
		10-15	84	42
		15-24	0	84
III	RS-III	1-24	84	0

[a] pH of the influent was adjusted to 8.50.

2.2.3. Analytical methods

TSS, VSS and SVI were determined according to Standard Methods (APHA et al., 2005). DO and temperature were measured with a digital portable dissolved oxygen meter (VWR DO 200, Belgium); pH with a digital pH-meter (Eutech Instruments Ecoscan pH 5, Belgium). Chl*a* was determined by centrifuging (HERMLE 2300 K, Germany) 5 mL of culture at 5000 g for 15 min at 10 °C, thereafter, adding 5 ml of 90 % acetone and 10 % $MgCO_3$ saturated solution and sonicating (5 min, cycle time 0.7, amplitude 70 %, dr. Hielscher Ultrasonic Processor UP 400 S, Germany) in order to disintegrate the cell walls. After allowing the samples to stand for 3 days at 7 °C, they were centrifuged at 5000 g for 15 min. Hereafter, the optical densities were measured with a UV-visible spectrophotometer (Shimadzu UV-1601, Japan) to determine pigment content following the methods and calculations in accordance with APHA et al. (2005). Each TSS, VSS and Chl*a* determination was performed in duplicate. The autotrophic index (AI), which represents the quotient between the VSS (mg L^{-1}) and Chl*a* (mg L^{-1}), was calculated in accordance to APHA et al. (2005). The microscopic observations were performed with an optical microscope (Reichert Neovar 300422, Austria). The filament count was assessed according to Jenkins et al. (2004). Floc size analysis was performed using a MSX17 automated wet sample dispersion unit (Malvern, UK) and a Mastersizer/S (Malvern, UK). Mean particle size D[4,3], i.e. the volume-weighted average floc diameter also known as mass mean diameter, was calculated according to Govoreanu et al. (2003).

Effluent samples were immediately filtered through a 0.20 µm pore size syringe filter (Chromafil RC-20125, Germany). $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ were determined on a 10 mL filtered sample by a Metrohm 761 Compact Ion Chromatograph as described by De Schryver and Verstraete (2009). The SCOD was measured spectrophotometrically with Hach Lange kits (Hach Lange DR 2800, Belgium). Turbidity of non-filtered effluent samples was measured with a portable turbidity meter (Hanna Instruments HI 93703, Germany).

2.2.4. Statistical analyses

Statistical analyses were performed using PASW Statistics 17.0 software (SPSS Incorporated, Chicago, Illinois). Normality of the data and homogeneity of variances were determined with a Kolmogorov Smirnov test and Levene's test respectively. If normal distribution and homogeneity of variances were observed, the significant differences between mean values were analysed by One-way ANOVA and a Tukey's post-hoc test ($p < 0.05$). If no normal distribution was observed, the differences in the means were statistically analysed by Kruskal-Wallis followed by a Mann-Whitney post-hoc test including a Bonferroni correction ($p < 0.05$). All correlations were quantified by Spearman's rho (r_s) in case of data was not normal distributed and with Pearson (r_p) for normal distributed data (2-tailed significance). All means are given as mean with standard deviation (number of measurements).

2.3. Results

2.3.1. Autotrophic index of the floc

The carbon source in the influent had a clear effect on the AI and thus on the Chl*a* content of the microalgal bacterial biomass (Fig. 2.1.). The AI of the MaB-flocs of RB-I and RB-II (reactor fed with bicarbonate in experimental run I or II, respectively) was significantly lower than the AI of the MaB-flocs of RS-I and RS-II (reactors fed with sucrose in experimental run I or II, respectively): 62.2 and 70.7 compared to 338.2 and 124.3, respectively. The AI of the MaB-flocs of RBS-I and RBS-II (reactors fed with bicarbonate and sucrose in experimental run I or II, respectively) ranged in between and was significantly lower than RS-I and RS-II and significantly higher than RB-I and RB-II. The AI was negatively correlated with the concentration of bicarbonate in the influent and positively correlated with the sucrose

concentration in the influent (r_p=±0.847 for all data of both experimental runs; $p < 0.001$; N=33). An average Chl*a* content of 3.0 ± 0.3, 8.1 ± 0.4, 16.3 ± 1.9 and 14.2 ± 1.1 mg Chl*a* g^{-1} VSS was observed in RS-I, RS-II, RB-I and RB-II, respectively. Microscopic observations confirmed a higher amount of photosynthetic microorganisms (PM) in the flocs of the reactors fed with bicarbonate compared to the reactors fed with sucrose.

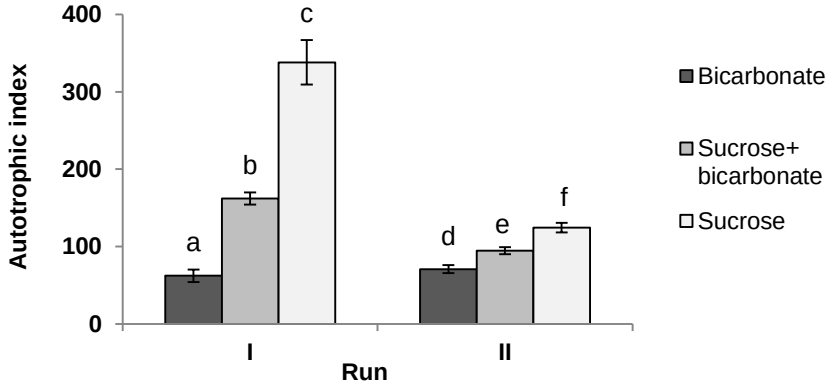


Fig 2.1. *Influence of the type of carbon source (inorganic/organic) on the autotrophic index of MaB-flocs in three MaB-floc SBRs fed with synthetic wastewater containing 84, 42 and 0 mg C-$KHCO_3$ L^{-1} and 0, 42, 84 mg C-sucrose L^{-1}, respectively (average and standard deviation of each experimental run)*

The values indicated with a different label are significantly different from each other within the same run according to a one-way ANOVA and a Tukey HSD post-hoc test ($p < 0.05$).

2.3.2. Floc settleability and structure

The formation of settable MaB-flocs consisting of activated sludge enriched with PM was feasible in lab-scale SBR reactors, allowing an easy separation of the microalgal bacterial biomass by settling. The D[4,3] of the MaB-flocs ranged from 148 µm to 305 µm in all experiments. Microscopic observations showed an incorporation of PM in the flocs (Fig. 2.2.). The predominant eukaryotic microalgae were *Chlorella* sp. However, the type of carbon source had a clear effect on the floc settleability, evaluated by means of SVI. In Figure 2.3.a, the data of experimental run I is shown as an example. The SVI in the second experiment showed a similar trend (data not shown). Whereas the SVI of the MaB-flocs of RS-I remained stable around 76 ± 15 mL g^{-1} TSS, the SVI of the MaB-flocs of RBS-I increased up to 628 mL g^{-1} TSS after 28 days (Fig. 2.3.a) and of RBS-II up to 990 mL g^{-1} TSS after 19 days (data

not shown), leading to a wash out of the biomass. The settleability of the flocs of the reactor fed with bicarbonate without sucrose decreased even faster. At day 11, the SVI increased to 671 mL g^{-1} TSS in RB-I and at day 15, the biomass of this reactor washed out due to a low settleability and bulking of the flocs which led to a higher turbidity of the effluent (Fig. 2.3.b). Therefore the SVI from RB-I could not be measured after day 15. At day 25, the biomass of RBS-I washed out, also resulting in a higher turbidity of the effluent (Fig. 2.3.b). The effluent turbidity was significantly higher in RB-I and RB-II compared to RBS-I and RBS-II, and compared to RS-I and RS-II (Table 2.3.).

Microscopic examination showed that filamentous phototrophic *Phormidium* species were strongly present in the flocs in the bicarbonate fed SBRs when the biomass of the reactors washed out. In contrast, filamentous organisms were hardly present in the RS-I and RS-II. Thus, the increasing SVI was consistent with the increasing levels of filamentous microorganisms (Fig. 2.2.).

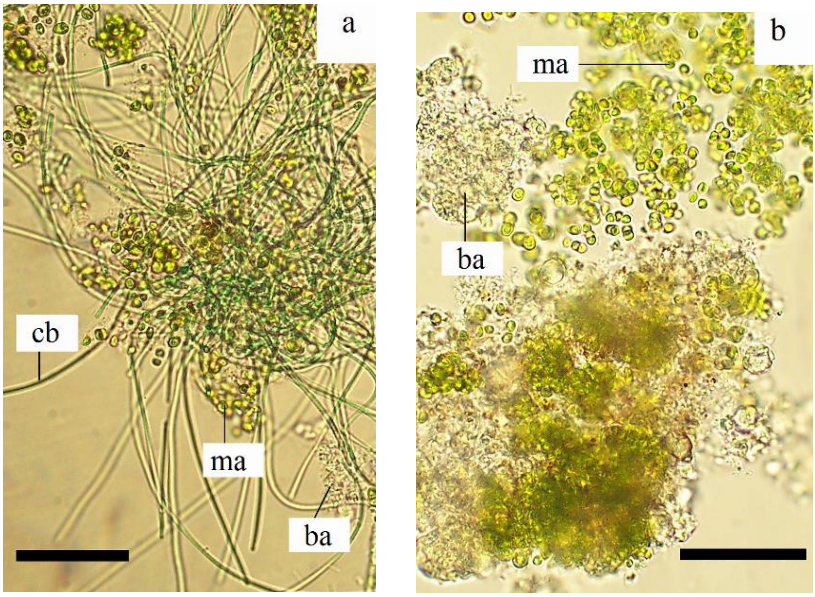


Fig. 2.2. *Light microscopy images (400x) of MaB-flocs from (a) bicarbonate fed reactor RB-I and (b) sucrose fed reactor RS-I (day 11) showing microalgae (ma), bacteria (ba) and filamentous cyanobacteria (cb)*
Depicted scale bars measure 50 μm in length.

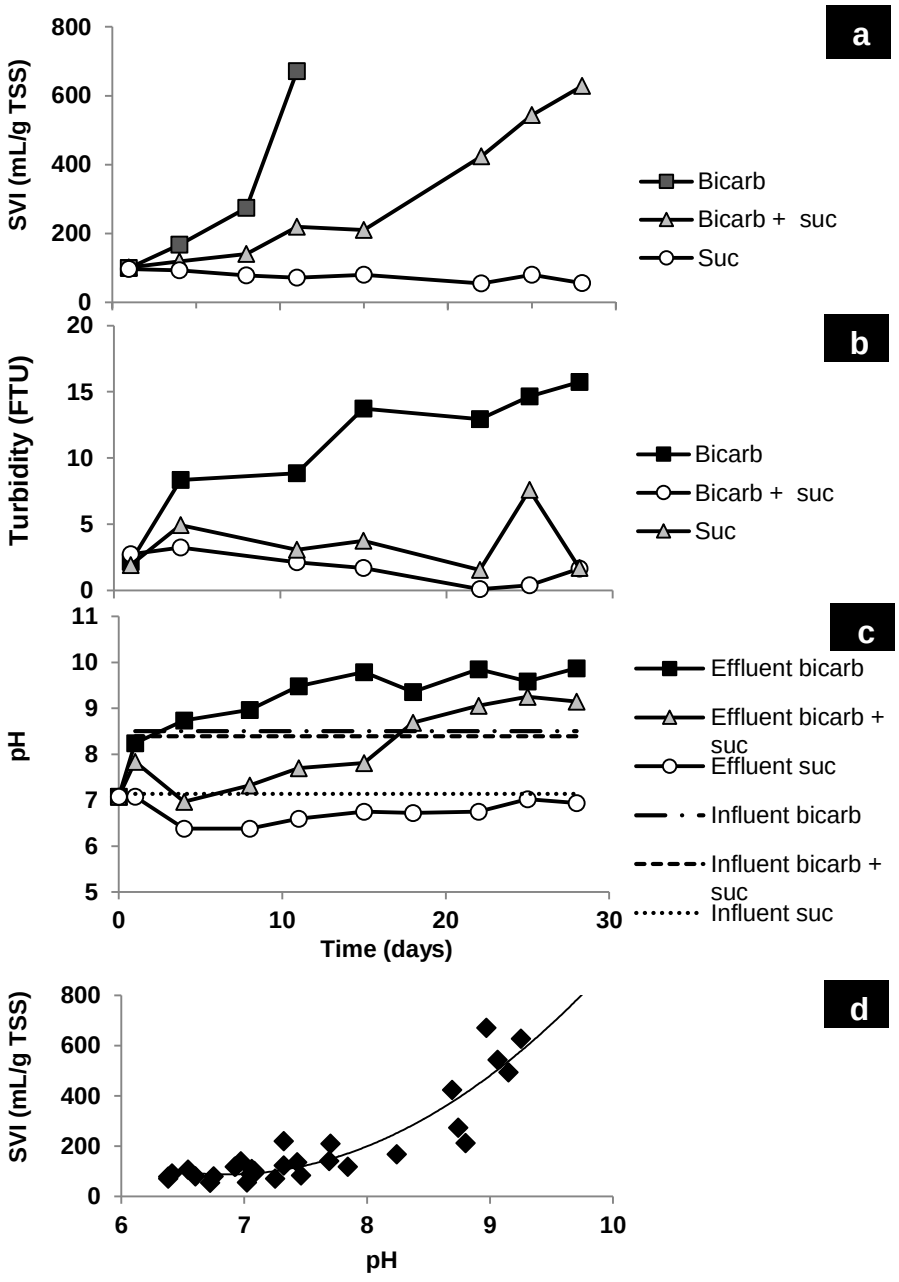


Fig. 2.3*. Influence of the type of carbon source (inorganic/organic) on MaB-flocs SBRs fed with synthetic wastewater containing 84, 42 and 0 mg C-$KHCO_3$ L^{-1} and 0, 42, 84 mg C-sucrose L^{-1} respectively: (a) SVI, (b) turbidity of effluent, (c) pH of influent (means of 4 measurements) and effluent during the first experimental run and (d) quadratic correlation (R^2= 0.85) between SVI and pH (values of two experimental runs)*

2.3.3. Evolution of reactor pH and dissolved oxygen

A shift in reactor pH was noticed. Reactors fed with only bicarbonate shifted up to nearly 3 log units, from around 7 to nearly 10 (Fig. 2.3.c). This increase was found to be smaller for reactors fed with both bicarbonate and sucrose compared to the SBRs fed with only bicarbonate. As SVI shifted simultaneously with pH, a significant quadratic correlation between the pH and the SVI could be observed (R^2 = 0.845; $p < 0.001$; N = 33; data of two experimental runs) (Fig. 2.3.d).

In all reactors, the DO level in the reactors increased in time during the illuminated reaction phases. In the RB-I and RB-II DO concentrations were measured between 108.6 % and 122.9 %, in RS-I and RS-II between 6.4 % and 28.7% and in RBS-I and RBS-II between 65.6 % and 87.1 % saturation. Therefore, the carbon source had a significant effect on the DO level in the reactor.

2.3.4. Reactor performance for SCOD and nitrogen removal

For all SBRs fed with organic carbon, a very high daily removal of SCOD per reactor volume was obtained (Table 2.3.): around 95 % in RS and around 93 % in RBS.

For experimental run I and II, the nitrate removal efficiency (NRE) was significantly lower in the SBRs fed with bicarbonate without sucrose compared to the other SBRs (Table 2.3). Hereby, the nitrate removal rate was 20.4 mg N L^{-1} day^{-1} in run I and 18.4 mg N L^{-1} day^{-1} in run II. The N RE of the reactors fed with bicarbonate and sucrose ranged between the values for the organic fed reactors and inorganic fed reactors (Table 2.3.). The nitrite concentrations of the effluent were below 1.1 mg N-NO_2^- L^{-1} during all experiments (data not shown).

Table 2.3. *Influence of bicarbonate and sucrose on the reactor performance of MaB-floc SBRs*

Run	Reac-tor[1]	Removal rate per g VSS (mg g^{-1} VSS day^{-1})		Removal rate per reactor (mg L^{-1} day^{-1})		Removal efficiency (%)		Effluent turbidity (FTU)
		SCOD[2]	$N\text{-}NO_3^-$	SCOD[2]	$N\text{-}NO_3^-$	SCOD[2]	$N\text{-}NO^{3-}$	
I	RB-I[3]	-	8.2 ± 5.8 $(4)^{c}$	-	3.3 ± 2.2 $(4)^{g}$	-	11.1 ± 7.4 $(4)^{g}$	8.6 ± 3.4 $(9)^{g}$
	RBS-I	135 ± 22 $(8)^{a}$	20.4 ± 6.7 $(8)^{c}$	154 ± 5 $(8)^{e}$	15.6 ± 4.3 $(8)^{h}$	92.3 ± 3.1 $(8)^{a}$	52.2 ± 14.4 $(8)^{h}$	1.5 ± 1.2 $(9)^{h}$
	RS-I	233 ± 57 $(8)^{b}$	22.6 ± 7.6 $(8)^{c}$	318 ± 6 $(8)^{f}$	20.4 ± 5.0 $(8)^{i}$	95.2 ± 1.8 $(8)^{b}$	68.2 ± 16.7 $(8)^{i}$	2.3 ± 1.6 $(9)^{i}$
II	RB-II[3]	-	11.1 ± 5.4 $(5)^{c}$	-	5.8 ± 2.6 $(5)^{g}$	-	19.7 ± 10.1 $(5)^{g}$	10.9 ± 4.8 $(7)^{g}$
	RBS-II	232 ± 104 $(7)^{a}$	23.8 ± 14.5 $(9)^{cd}$	158 ± 7 $(7)^{e}$	11.3 ± 1.6 $(9)^{h}$	94.2 ± 4.1 $(7)^{a}$	37.9 ± 5.2 $(9)^{h}$	1.7 ± 1.2 $(7)^{h}$
	RS-II	225 ± 21 $(7)^{a}$	19.4 ± 6.8 $(9)^{d}$	320 ± 7 $(7)^{f}$	18.4 ± 6.8 $(9)^{i}$	95.7 ± 2.0 $(7)^{a}$	61.4 ± 22.6 $(9)^{i}$	3.5 ± 2.2 $(7)^{i}$

[1] Carbon composition of the influent: RB: 84 mg C-bicarbonate L^{-1}; RBS: 42 mg C-bicarbonate L^{-1} and 42 mg C-sucrose L^{-1}; RS: 84 mg C-sucrose L^{-1}; [2] Shown where relevant, i.e. where organic carbon was fed to the reactor; [3] Only N-removal data before biomass wash out was used; [a–b] The values indicated with another superscript are significantly different from each other within the same experimental run according to a t- test ($p < 0.05$); [c-d] The ranges indicated with another superscript are significantly different from each other within the same experimental run according to Kruskal-Wallis test ($p < 0.05$) followed by a Mann-Whitney test with Bonferroni correction ($p < 0.05$); [e-f] The values indicated with another superscript are significantly different from each other within the same experimental run according to a t-test ($p < 0.05$); [g-i] The ranges indicated with another superscript are significantly different from each other within the same experimental run according to Kruskal-Wallis test ($p < 0.05$) followed by a Man-Whitney test with Bonferroni correction ($p < 0.05$).

2.3.5. Relationship pH, bicarbonate concentration, SVI and nitrogen removal

To verify whether the differences in reactor performance and floc characteristics were due to a higher pH or to the bicarbonate loading rate, a third experimental run was performed (Table 2.2.). When the RBSP-III was fed with 84 mg C-bicarbonate L^{-1}, an increase of the SVI and turbidity accompanied by a decrease of the nitrogen removal efficiency was observed compared to RS-III (Fig. 2.4.). This is in line with the previous experimental runs. This effect was not observed with a higher influent pH. The filament counts confirmed the increase of filamentous organisms in the RBSP-III after adding bicarbonate (28.9 compared to 3.8 filament counts per µL at day 20 and 36.8 compared to 5.1 counts per µL at day 24, for the RBSP-III compared to RS-III respectively).

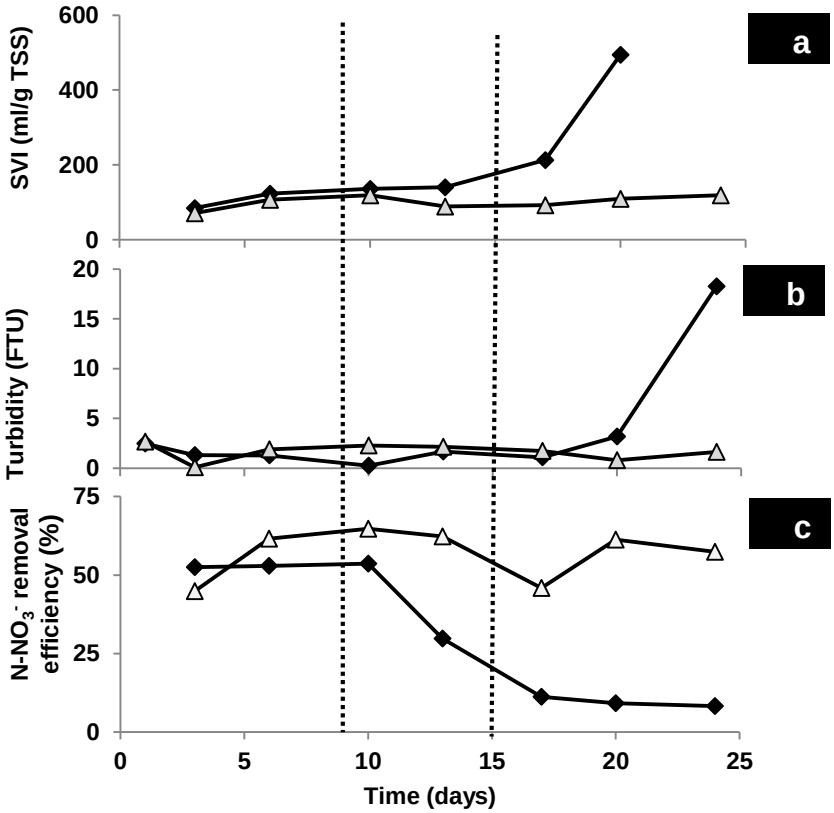


Fig. 2.4. *Sludge volume index (SVI) (a), effluent turbidity (b) and nitrate removal efficiency (c) of two MaB-floc SBRs: (1) fed with 84 mg C-sucrose L^{-1} (△); (2) fed on day 1-9 with 84 mg C-sucrose L^{-1} with a pH adjusted to 8.5, on day 10-15 with 42 mg C-sucrose L^{-1} and 42 mg C-bicarbonate L^{-1}, and on day 16-24 with 84 mg C-bicarbonate L^{-1} (◆)*

2.4. Discussion

In the present study, clear differences were found both in floc characteristics (AI and floc settleability) as in reactor performance (pH, SCOD removal, dissolved oxygen and nitrogen removal) of MaB-floc SBRs fed with organic carbon compared to inorganic carbon.

2.4.1. Autotrophic index and chlorophyll *a* content

The AI of the MaB-flocs was lower when bicarbonate was added compared to sucrose. In a microalgal bacterial community, lower AI values indicate autotrophic dominance and higher values indicate heterotrophic dominance (APHA et al., 2005). Despite the different inocula, this same trend was seen in experiment I as in experiment II.

In general, the Chl*a* content is a poor indicator of the photosynthetic microorganisms (PM) content, since it varies with the light availability (depending on the photon flux density provided, cell density and mixing) (Geider, 1987). However, in this study, the latter were kept constant, thus comparing in between reactors is possible. Our results suggest that bicarbonate favours the growth of PM in the floc, while sucrose favours the growth of non-photosynthetic bacteria in the floc. Thus, bicarbonate can be used to increase the incorporation of PM in MaB-flocs. The Chl*a* content of the MaB-flocs fed with organic carbon (3.0–8.1 g Chl*a* g^{-1} VSS) was lower compared to those reported by Gutzeit et al. (2005), i.e. 13.6 – 23.5 g Chl*a* g^{-1} VS, and Medina and Neis (2007), i.e. 3.1 - 10.3 g Chl *a* g^{-1} suspended solids; both for microalgal bacterial flocs consisting of activated sludge and *Chlorella vulgaris*, all fed with organic carbon without added inorganic carbon. This might be explained by the lower HRT applied in this study compared to these authors: 0.67 *vs* 2.7 or 4.0 days. Medina and Neis (2007) found that significantly lower Chl*a* concentrations were reached for lower HRTs.

2.4.2. Floc settleability

A good floc settleability is a crucial factor for the success of this MaB-floc system for wastewater treatment. The poor settling flocs in the bicarbonate fed reactors in all three experiments was due to the strong presence of filamentous cyanobacteria (*Phormidium* sp.). Cyanobacteria have a higher half saturation constant

$K_{m(CO2)}$ – the concentration of substrate at which the reaction occurs at one half its maximal rate – than microalgae, showing their relatively lower CO_2 binding capacity (Graham and Wilcox, 2000) but can also take up carbonate next to bicarbonate (Mikhodyuk et al., 2008). This might explain the increase in abundance of filamentous cyanobacteria in MaB-flocs when bicarbonate was added to the reactor accompanied by a pH increase. From a pH of 9 and higher, inorganic carbon is present as carbonate and bicarbonate, favouring the growth of these filamentous cyanobacteria and thus increasing the SVI. The strong correlation between SVI and pH found in our experiments support this hypothesis. This was confirmed with the third experimental run, where the SVI, turbidity and filament counts increased as soon as bicarbonate was added to the reactor, but not when NaOH was added. In contrast, filamentous microorganisms were hardly present in sucrose fed reactors, resulting in a stable reactor.

Toxic cyanobacteria are of concern, since the treated effluent containing cyanotoxins will be discharged into water bodies and can affect human and animal health (Avaygan, 2010; Furtado *et al*., 2009). While *Arthospira* sp. (Spirulina) has been on the food market for several decennia and some other cyanobacteria such as *Phormidium* sp. have been suggested for wastewater treatment (Sylvestre *et al*., 1996; Laliberté *et al*., 1997; Karacakaya *et al*., 2009) and as therapeutic agents for their anti-tumor activity, some cyanobacteria produce cyanotoxins (Teneva *et al*., 2005). Certain *Phormidium* species can produce hepatoxins (microcystins) or neurotoxins (anatoxins, saxitoxin, β-N-methylamino alanine) (Fristachi and Sinclair, 2005; Teneva *et al*., 2005). The cyanotoxin production was out of scope of this study, but are of concern and should be investigated in an experimental setup with real wastewater. Nevertheless, this study showed that a lower IC:OC ratio could prevent the abundance of filamentous cyanobacteria (*Phormidium* sp.) in the MaB-floc, as seen from the lower filament index and microscopic observations. This finding is of major importance in avoiding potentially toxigenic cyanobacteria in biological wastewater treatment (Avaygan, 2010).

2.4.3. pH

The pH increased in bicarbonate fed reactors during the light phases by the photosynthetic inorganic carbon uptake (Becker, 2008). In the reactors fed with only

organic carbon, the pH remained neutral. This could be attributed to (1) the lower PM and higher bacteria content of the flocs and (2) the acidification of sucrose metabolism. The drawbacks of a high pH were clearly present in our study: increased cyanobacterial growth, a high SVI and finally reactor washout. It is difficult to delineate the direct effect of pH on cyanobacterial growth from other possible more indirect pH effects such as shifts in the carbonate/bicarbonate balance or micronutrients availability. However, for guaranteeing a stable performance, a low pH should be maintained by simplementing this technology for wastewaters with a balanced IC:OC ratio.

2.4.4. Organic carbon removal by photosynthetic oxygen

In this study, a similar daily SCOD removal was obtained as in sewage fed microalgal bacterial reactors (Gutzeit et al., 2005), but with a 10 times lower PPFD of 100 µmol PAR photons m^{-2} s^{-1}. Gutzeit et al. (2005) recorded a maximum daily COD removal of 420 mg $L_{influent}^{-1}$, accounting for a COD removal of 280 mg $L_{reactor}^{-1}$ day^{-1}. The high SCOD removal efficiencies of up to 95 % obtained in this study suggest that this low PPFD was sufficient for the photosynthetic aeration of the used wastewater. Daily, 154 mmol PAR photons $L_{reactor}^{-1}$ were provided. This means that, assuming a realistic quantum yield of 0.08 (Emerson and Lewis, 1943), maximum 12.3 mmol O_2 $L_{reactor}^{-1}$ day^{-1} could be produced by photosynthesis, being more than the measured sCOD removal of 10.0 mmol O_2 $L_{reactor}^{-1}$ day^{-1}, and thus could be sufficient. Our results are in line with the findings of Gutzeit et al. (2005), stating that the oxygen produced by microalgal photosynthesis is accumulated within the aggregates and, thus, can be very efficiently used by bacteria. Such quasi equilibrium of microalgal and bacterial growth obviously promotes the formation of microalgal bacterial flocs (Gutzeit et al., 2005). Also Tison and Lingg (1979) found that *C. pyrenoidosa* could support the O_2 demand for the used bacterial community. However, previously, photosynthetic O_2 production has been reported as a limiting factor in microalgal bacterial systems (Guieysse et al., 2002; Muñoz and Guieysse, 2006) and was attributed to the general lower growth rate of microalgae compared to heterotrophic bacteria due to their larger size (Fenchel, 1974).

The SCOD removal per gram VSS was high and similar compared to that in a conventional aerated activated sludge reactor in a municipal wastewater treatment

system (Chan et al., 2009). However, our SCOD removal rate of around 300 mg COD L^{-1} day^{-1} was rather low compared to mechanical aerated activated sludge systems. For example, Sperling *et al.* (2001) reported a higher removal rate of 673-1672 mg COD L^{-1} day^{-1} with aerated activated sludge, from municipal wastewater containing 386-958 mg COD L^{-1} with a HRT of 6.8 h. To increase this, a higher COD loading rate combined with a higher photosynthetic photon flux density and/or lower HRT could be applied.

2.4.5. Nitrogen removal

Possible nitrate removal pathways in microalgal bacterial reactors are microalgal and bacterial uptake and denitrification. Our data suggests that nitrate uptake by microalgae contributed little to the nitrogen removal, since the nitrate removal was significantly higher in the organic carbon reactors where bacteria were dominating compared to the bicarbonate fed reactors where PM were dominating. Denitrification could not have played a major role in this nitrate removal, since (1) the herefore needed denitrification rate of 20.4 g N g^{-1} TSS h^{-1} is a rather high number (Onnis-Hayden and Gu, 2008) and (2) our results for nitrogen removal are in the same order as obtained by Gutzeit et al. (2005), i.e. 33.2 mg L^{-1} day^{-1} with a continuous microalgal bacterial system where no denitrification was observed. This suggests that the main driving force for the nitrate removal by MaB-flocs was microbial assimilation. Since nearly all SCOD was removed in the sucrose fed reactors, the SCOD was probably limiting the bacterial nitrogen uptake and, hence, higher nitrogen removal might be feasible with a higher SCOD loading rate. This should be confirmed in future experiments.

In this study, the daily nitrogen removal rates were comparable to those for municipal wastewater treated with aerobic activated sludge (Kim et al., 2009), but lower than with bio-flocs in SBRs (De Schryver and Verstraete, 2009), and higher than with *Phormidium laminosum* immobilized on hollow fibers in a photobioreactor (Sawayama et al., 1998). The COD and N removal and photosynthetic aeration (and thus CO_2 assimilation) of our study demonstrated the great potential for the application of MaB-floc reactors for environmental technology.

2.5. Conclusions and outlook

To screen the potential of microalgal bacterial flocs (MaB-flocs) for wastewater treatment, the influence of an inorganic and organic carbon source on the reactor performance was investigated. The following conclusions were reached.

- The inorganic carbon source bicarbonate significantly increased the chlorophyll *a* concentration of the MaB-flocs, but only poor settling flocs could be obtained resulting in a high turbidity of the effluent. Moreover, the pH increased to 9.5 and low nitrogen removal efficiencies were measured.
- The organic carbon source sucrose resulted in good settling MaB-flocs, high nitrogen removal efficiencies and neutral pH levels. Despite a lower chlorophyll *a* content of the biomass and the lower *in situ* oxygen concentration, high SCOD removal efficiencies of 95 % were achieved when sucrose was added.

This study shows the need for an organic carbon source for a good MaB-floc reactor performance. The bicarbonate and organic carbon concentration of the wastewater is of major importance when MaB-flocs are to be used for wastewater treatment.

Despite the numerous advantages of this wastewater treatment with respect to greenhouse gas mitigation and energy saving due to no need for mechanical aeration, the intrinsic land and light requirements might hinder its widespread implementation on large scale. Compared to an activated sludge reactor, the additional investment cost will be mainly coming from the ground use, since a high surface to volume ratio is needed for microalgal reactors (van Beilen, 2009). Therefore, to further maximize the efficiency of the process and make this MaB-floc system a practical reality, more research is needed. This should include (1) incorporation in MaB-flocs of microalgal species which have high CO_2 fixing and high solar energy conversion efficiencies at low light intensities and (2) MaB-flocs which have higher nutrient removal rates.

Next to an optimal IC:OC ratio of wastewater, the C:N ratio of wastewater should be high enough to enable scavenging all N from the wastewater by MaB-flocs. This C:N ratio can be increased by CO_2 addition via flue gas sparging. However, flue gas addition will also alter the IC:OC ratio. Therefore, it should be investigated whether flue gas sparging could enhance wastewater treatment without negatively affecting the MaB-floc settleability.

2.6. Acknowledgements

This work was supported by an academic Ph.D. grant from Howest University College West-Flanders and UGent. The authors thank J. Arends, J. Benner, W. De Muynck, J. Schouppe and D. van der Ha (UGent) for their useful suggestions; K. Muylaert (Kuleuven) for the microscopic determination of the photosynthetic microorganisms; G. Van de Velde (UGent), and G. Bellens, A.Vergote and B. Willems (Howest) for their assistance during the practical work.

$$-1 \times -1 = +1$$

$$-2 \times -1 = +2$$

$$-3 \times -1 = +3$$

Multiplying two negative numbers, always results in a positive number.

(Magrhibi, 1130-1180)

Quote on previous page:
Mathematical convention presented by Al-Samaw'al Maghribi in the 12th Century in his book 'al-Bahir fi'l-jabr', meaning 'the brilliant in algebra'.

CHAPTER 3

Bioflocculation of microalgae and bacteria combined with flue gas to improve sewage treatment

Abstract

Although microalgae are promising for a cradle-to-cradle design approach of sewage treatment, their application is hampered by high harvesting costs and low C:N ratios of sewage. Therefore, the potential of microalgal bacterial flocs (MaB-flocs) were investigated for the secondary treatment of sewage supplemented with different flue gas flow rates (FGFRs) from a coal power plant. Effluent (N, P, turbidity and pH) and off gas discharge levels (NO_x, SO_x) met the European discharge limits with a hydraulic retention time of only 0.67 days and a FGFR of 0.6 L h^{-1} (0.0025 vvm). This FGFR provided sufficient carbon and resulted in removal efficiencies of 48 ± 7 % CO_2, 87 ± 5 % NO_x and 99 ± 1 % SO_2. MaB-flocs settled fast reaching up to a density of 19 g VSS L^{-1}. High biomass productivities (0.18 g VSS L^{-1} day^{-1}) were obtained under a low light intensity. This successful reactor performance indicates the large potential for the industrial application of MaB-flocs for flue gas sparged sewage treatment.

Chapter redrafted after:

Van Den Hende, S., Vervaeren, H., Desmet, S., Boon, N., 2011a. Bioflocculation of microalgae and bacteria combined with flue gas to improve sewage treatment. New Biotechnol. 29, 24-31.

3.1. Introduction

Due to the world-wide issues regarding depletion of resources and global warming, there is a true renaissance of interest in microalgae for environmental technologies. Currently, biological sewage treatment is mainly performed in conventional activated sludge systems, where up to 50 % of the energy is used for mechanical aeration (Tchobanoglous et al., 2003) to convert COD into the greenhouse gas CO_2, while nitrogen is 'wasted' by nitrification/denitrification. A sustainable redesign of sewage treatment is emerging where nutrients act as a bioresource rather than a waste material (Verstraete et al., 2009), akin to natural ecosystems where nutrients and energy are recycled. Microalgae could play a key role in this redesign. Being photosynthetic microorganisms, they can lower the emission of CO_2, lower the need for mechanical aeration by providing oxygen via photosynthesis, scavenge resources (C, N and P), and convert solar energy into biomass.

To maximise scavenging of resources from sewage, flue gas should be supplemented to the sewage for different reasons. First, domestic sewage typically contains insufficient carbon for a maximal N and P scavenging with microalgae: the C:N ratio of 3-7 in domestic sewage (Tchobanoglous et al., 2003) is low compared to a C:N ratio of 3-17 in microalgal biomass (Geider and La Roche, 2002). Second, an increased photosynthesis and thus solar energy harnessing may be expected by CO_2 supplementation to sewage (Park and Craggs, 2010b). Third, the addition of flue gas optimizes the reactor pH, counteracting the increasing pH due to microalgal autotrophic growth. This avoids ammonium volatilization and keeps the pH within the optimum biological growth range (Park et al., 2011a). Fourth, the conversion of flue gas pollutants into biomass could deliver CO_2 and NO_x credits in certain countries. Fifth, to avoid C and N loss to the air via denitrification and anaerobic digestion, the reactor should be kept aerobic. Therefore, photosynthetic oxygen production should outrange bacterial oxidation of COD by providing extra inorganic carbon. Finally, the addition of flue gas may also contribute in reactor mixing and stripping of accumulated oxygen. However, the flue gas rate has to be limited to avoid algal toxicity to CO_2, NO_x and SO_x (Lee et al., 2000b) and to reach stringent off gas emission limits. Therefore, an optimum flue gas rate should be determined.

Microalgae for combined sewage treatment and flue gas treatment is not a new concept and has been introduced in scientific literature more than 5 decades ago

(Oswald and Golueke, 1960). Though, the amount of scientific publications on this microalgal technology greatly outnumbers the amount of its industrial implementations. The high cost of microalgae harvesting, up to 57 % of the total microalgae production cost, has been proven a major limitation of the implementation of microalgal ponds for sewage treatment (Richmond, 2004). Expensive techniques such as centrifugation, filtration, coagulation and/or flocculation should be replaced by cheaper alternatives (Uduman et al., 2010). A possible cheaper and more straightforward way of separating the biomass is bioflocculation. Microalgal bacterial flocs or MaB-flocs are aggregations of microalgae and bacteria that due to their larger size settle quickly by gravity (Van Den Hende et al., 2011b; Gutzeit et al., 2005).

The use of flocculating microalgal bacterial biomass for the treatment of wastewater combined with flue gas has been proposed by several researchers (Kumar et al., 2010; Pittman et al., 2010) as the most promising option to achieve both good effluent quality in terms of suspended solids and economically feasibility. However, as far as we know, no experimental data has been published yet. The present study evaluates the potential of MaB-flocs for the treatment of primary treated sewage combined with flue gas mimicking a coal burning process. It was examined whether a good effluent and off gas quality could be achieved while maintaining good floc properties in a lab-scale reactor. Moreover, the reactor performance was optimised by altering the flue gas flow rate (FGFR).

3.2. Materials and methods

3.2.1. MaB-floc cultivation

The microalgal bacterial flocs (MaB-flocs) were precultured as described by Van Den Hende et al. (2011) starting from: (1) a consortium of local photosynthetic microorganisms collected in the vicinity of a coal fired power plant (Electrabel, Ruien, Belgium), predominantly containing *Chlorella* sp., *Pediastrum* sp., *Phormidium* sp. and *Scenedesmus* sp., and (2) aerobic activated sludge from the aeration basin of a municipal wastewater treatment plant (Aquafin, Harelbeke, Belgium).

3.2.2. Wastewater and flue gas

Primary treated sewage was collected just after the sand and grease trap of a municipal wastewater treatment plant (Aquafin, Harelbeke, Belgium) and used as

influent. This influent was sieved (1 mm) prior to feeding and stored at 4 °C. It contained (in mg L^{-1}): 44.30 ± 5.17 total nitrogen (TN), 37.27 ± 7.92 $N\text{-}NH_4^+$, 0.01 ± 0.07 mg $N\text{-}NO_2^-$, 0.06 ± 0.04 $N\text{-}NO_3^-$, 68.5 ± 16.4 total inorganic carbon (TIC), 57.2 ± 22.0 total organic carbon (TOC), 1.39 ± 0.91 $P\text{-}PO_4^{3-}$ and 24.85 ± 8.66 $S\text{-}SO_4^{2-}$, and had a pH of 7.46 ± 0.18, a turbidity of 67.6 ±16.8 FTU and a molar C:N ratio of 4.22 ± 2.14.The injected flue gas contained 214 ± 4 g CO_2 Nm^{-3}, 383-397 mg NO Nm^{-3} and 572 ± 11mg SO_2 Nm^{-3} with the balance N_2 (Lindegas, Belgium), mimicking flue gas from a coal burning power plant (Xu et al., 2003).

3.2.3. Reactor set-up

Experiments were performed in a gastight, Plexiglass, counter current, bubble column photobioreactor of 5 L with a working volume of 4 L and an illuminated surface area of 0.136 m^2. Lamps (2 TL-lamps of 55 Watt, Philips, Belgium; 1 fog of 500 Watt, Massive, Belgium) provided a photosynthetic photon flux density (PPFD) of 100 ± 4 µmol PAR photons m^{-2} s^{-1} (Field Scout Quantum Light Meter, USA) at the inner reactor wall. Three pumps were connected to the reactor: a peristaltic pump for the circulation of the MaB-flocs (counter flow rate: 20 L h^{-1}; Watson Marlow, USA), a diaphragm pump for the influent feeding (Blackstone, USA) and a peristaltic pump for the effluent wastewater withdrawal (Watson Marlow, USA) (Fig. 3.1.). The reactor was operated at 20 ± 3 °C in sequencing batch modus. Three cycles of 8 hours were applied every 24 hours and each cycle consisted of reaction (6.5 h), settling (0.5 h), effluent withdrawal (0.5 h) and influent feeding (0.5 h) (Fig. 3.1.). At the end of every cycle 2 litres of effluent was replaced by 2 litres of influent, resulting in a hydraulic retention time (HRT) of 0.67 days. When needed, just before the settling phase the reactor liquor was harvested to obtain a MaB-floc density of 1 g VSS L^{-1}. The flue gas flow rate was set by a flow controller (Supelco, Belgium) and a magnetic valve (Norgren, Belgium). All tubing was gastight (Tygon, Belgium; Saldaform, Belgium).

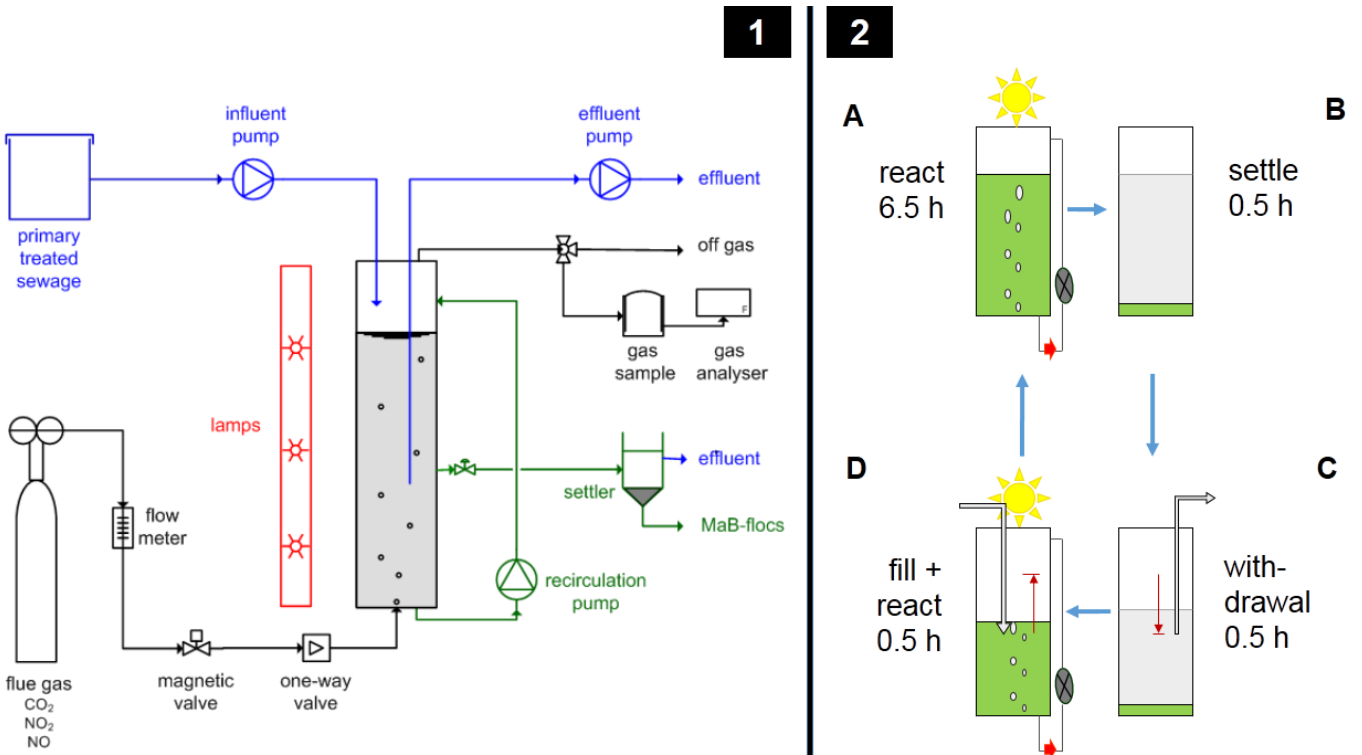


Fig. 3.1. *MaB-floc reactor set-up for the combined treatment of sewage and flue gas (1) and its SBR modus during an 8 hour cycle consisting of 4 phases (2): reaction time with flue gas sparging, reactor mixing and irradiation (A), settling time (B), 2 L effluent withdrawal (C) and 2 L influent addition while flue gas sparging, reactor mixing and irradiation (D)*

Different FGFRs (1.2 L h^{-1} or 0.0050 vvm in $MaB_{1.2\ L}$; volume of gas per volume of mixed liquor per minute; 0.6 L h^{-1} or 0.0025 vvm in $MaB_{0.6\ L}$; 0.0 L h^{-1} or 0.0 vvm in $MaB_{0.0\ L}$) were applied and compared to the controls NoMaB and RH_2O (Table 3.1.). No additional aeration was performed, therefore restricting oxygen supply to *in situ* biological oxygen production. All feeding, recirculation, decanting, stirring, gas flow and irradiation were controlled with programmed timers.

Table 3.1. *Overview of the operation modes of MaB-flocs SBRs with different influent, flue gas flow rates (FGFRs), reactor liquor and operation periods*

Reactor	Influent	FGFR (L h^{-1})	Liquor	Operation period (days)
$MaB_{1.2\ L}$	Sewage	1.20	MaB-flocs	29
$MaB_{0.6\ L}$	Sewage	0.60	MaB-flocs	32
$MaB_{0.0\ L}$	Sewage	0.00	MaB-flocs	44
NoMaB[1]	Sewage	0.60	No MaB-flocs	19
RH_2O[2]	Deionised water	0.60	No MaB-flocs	4

[1] Served as control for $MaB_{0.6\ L}$ to investigate the effect of MaB-flocs on pollutant removal from sewage and flue gas; [2] Served as control for NoMaB to investigate the effect of sewage on effluent pH and flue gas removal.

3.2.4. Analytical protocols

The reactor liquor was sampled by collecting 100 mL liquor three times a week 2 hours before the settling phase of the second SBR cycle and analyzed for chlorophyll *a* (Chl *a)*, total suspended solids (TSS) and volatile suspended solids (VSS). Each TSS, VSS and Chl *a* determination was performed in duplicate and analyzed as described by Van Den Hende et al.(2011). To determine the physiological condition of the microalgae, the $A664_b$:$A665_a$ ratio of 3 mL Chl*a* and pheophytin*a* extract was measured ($A664_b$ is the absorbance at 664 nm before acidifying, and $A665_a$ is the absorbance at 665 nm after acidifying with 100 µL of 0.1 M HCl). The sludge volume index (SVI) was determined on the wasted microalgal bacterial biomass. The $A664_b$:$A665_a$ ratio, SVI and autotrophic index (AI) were determined according to Standard Methods (APHA et al., 2005). Microscopic observations were performed once a week with an optical microscope (400 x; Reichert Neovar 300422, Austria). The lipid and neutral lipid content of the MaB-flocs were analysed by a modified Bligh and Dyer method (1959), as described by Ryckebosch et al. (2011).

Effluent wastewater was sampled three times a week. A part was immediately filtered through a 0.20 µm pore size syringe filter (Chromafil RC-20125, Germany). Anion ($P\text{-}PO_4^{3-}$, $S\text{-}SO_4^{2-}$, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$) concentrations were determined by a Metrohm 761 Compact Ion Chromatograph (Van Den Hende et al., 2011b). TIC, TOC, TC, TN and $N\text{-}NH_4^+$ were measured spectrophotometrically with Hach Lange Kits (Hach Lange DR 2800, Belgium) on raw samples, except for $N\text{-}NH_4$ which was measured after filtering. Turbidity was measured with a portable microprocessor turbidity meter (Hanna Instruments HI 93703, Germany) and pH with a calibrated pH-meter (Eutech Instruments Ecoscan pH 5, Belgium). Three times a week the off gas concentrations were analysed by a flue gas analyser (TESTO 350, Germany; CO_2, CO, NO, NO_2, SO_2, O_2). These flue gas compound concentrations were recalculated to standard conditions of 273.15 K and 101.325 kPa.

3.2.5. Statistical analysis

Statistical analyses were performed using PASW Statistics 17.0 software (SPSS Incorporated, Chicago, Illinois). Normality of the data and homogeneity of variances were determined prior to any statistical treatments with a Kolmogorov Smirnov test and Levene's test respectively. Since no normal distribution was observed, the differences in the means were statistically analysed by Kruskal-Wallis followed by a Mann-Whitney post-hoc test including a Bonferroni correction ($p < 0.05$).

3.3. Results and discussion

3.3.1. Sewage treatment

The average concentrations of NH_4^+, NO_3^-, NO_2^- and TN of the sewage effluent of $MaB_{0.6\ L}$ (Fig. 3.2.a; Fig. 3.2.b) were lower than the European Standard (ES) of nitrogen for discharge of treated sewage in surface water. These ES are 15 mg TN L^{-1} (yearly average) for 10,000 – 100,000 population equivalents or 10 mg TN L^{-1} (yearly average) for > 100,000 populations equivalents (EU, 1991). In this reactor, the mean total nitrogen removal of 40.49 ± 5.49 mg TN $L_{reactor}^{-1}$ day^{-1} ($MaB_{0.6\ L}$) was nearly double than earlier reported with MaB-flocs in synthetic wastewater (Van Den Hende et al., 2011b; Gutzeit et al., 2005). Moreover, this was obtained with a low PPFD of 100 µmol PAR photons m^{-2} s^{-1} and a short HRT of 0.67 days. This HRT is 3

to 12 times shorter than previous reports on sewage treatment with microalgae. For example, Woertz et al. (2009) applied a HRT of 2-4 days, and Park and Craggs (2010), 4-8 days. Consequently, a smaller (maximum 3 to 12 times) microalgal pond area could be feasible with MaB-flocs, addressing one of the major drawbacks of microalgal cultures for sewage treatment.

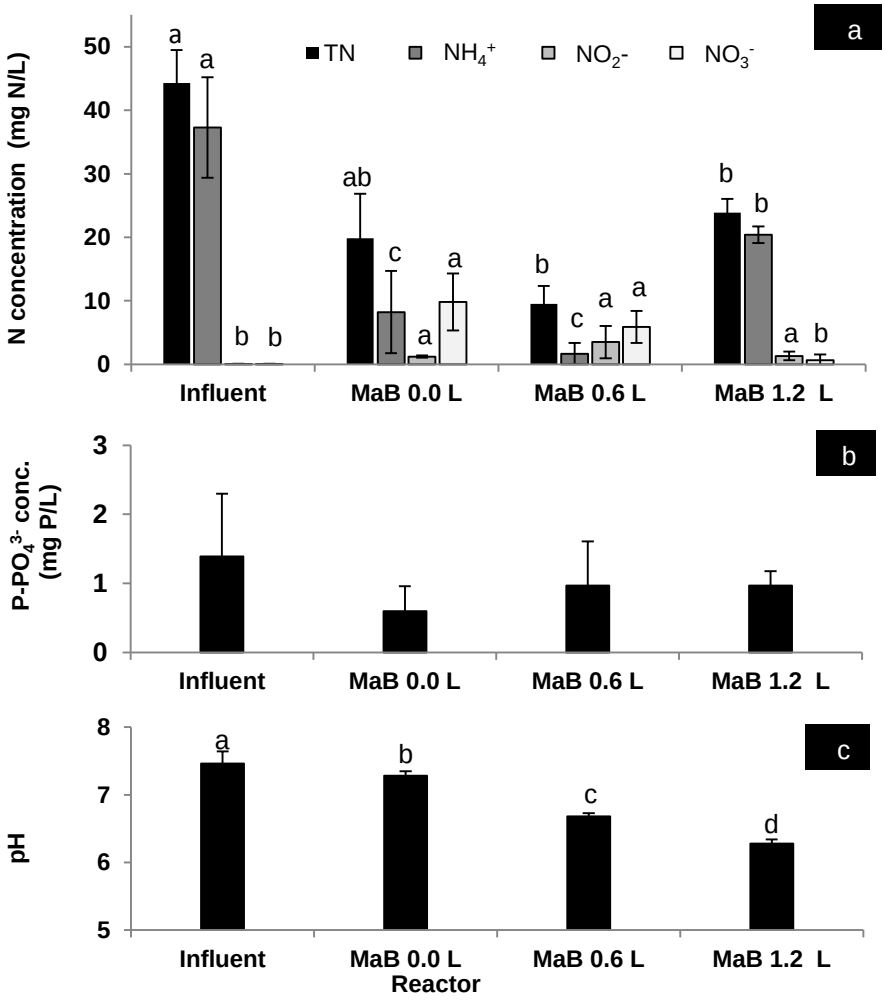


***Fig. 3.2**. Effluent characteristics of sewage fed MaB-floc SBRs supplemented with different flue gas flow rates: 0.0 L h^{-1} ($MaB_{0.0L}$), 0.6 L h^{-1} ($MaB_{0.6L}$), 1.2 L h^{-1} ($MaB_{1.2L}$) compared to influent: nitrogen concentration (a), orthophosphate concentration (b), and pH (c)*

The ranges indicated with a different superscript (a-c) within the same pollutant are significantly different from each other according to a Kruskal-Wallis test followed by a Mann-Whitney test with Bonferroni correction ($p < 0.05$).

The nitrate concentration of the effluent was significantly lower in $MaB_{1.2\ L}$ (0.75 ± 0.88 mg N L^{-1}) compared to $MaB_{0.6\ L}$ (5.89 ± 2.51 mg N L^{-1}) and $MaB_{0.0\ L}$ (9.81 ± 4.48 mg N L^{-1}; Fig. 3.2.a). This lower nitrate concentration in the reactor with the highest flue gas rate of 1.2 L h^{-1} could not be attributed to a removal of all nitrogen, since the ammonium concentration of the effluent in this reactor was even higher than in $MaB_{0.0\ L}$ (20.41 ± 1.32 and 8.23 ± 6.47 mg N-NH_4^+ L^{-1} resp.). These results suggest that flue gas sparging, containing CO_2, NO, SO_2 and N_2, could decrease the nitrification (leading to a higher ammonium and lower nitrate concentration in the effluent) and/or increase the nitrate uptake (leading to a lower nitrate concentration in the effluent) by MaB-flocs. The enhancement of nitrate reductase and nitrite reductase by NO, also needed by microalgae to convert nitrate into ammonium after nitrate uptake, has been reported for a terrestrial plant *Brassica* sp. (Du et al., 2008) but not for microalgae. Further research is needed to clarify whether flue gas sparging inhibits bacterial ammonium oxidation, enhances microalgal nitrate uptake and/or decreases the ammonium inhibition of nitrate uptake in light conditions (Yin et al., 1998).

The sewage effluent contained 0.60 ± 0.36, 0.97 ± 0.64 and 0.97 ± 0.21 mg P-PO_4^{3-} L^{-1} in $MaB_{0.0\ L}$, $MaB_{0.6\ L}$ and $MaB_{1.2\ L}$, respectively (Fig. 3.2.b). These concentrations were lower than the ES of 2 mg TP L^{-1} for discharge of treated urban wastewater (yearly average for 10 000 – 100 000 population equivalents; EU, 1991) suggesting that this ES can be reached. Little optimisation is needed to reach the more severe ES of 1 mg TP L^{-1} (yearly average for > 100 000 populations equivalents; EU, 1991). The highest removal of phosphate, 2.26 ± 0.92 mg P-PO_4^{3-} $L_{reactor}^{-1}$ day^{-1}, was observed in $MaB_{0.6\ L}$.

The mean pH of the sewage influent was 7.42 ± 0.06. In the control reactor with only deionised water (RH_2O), the pH decreased from 7.09 to 5.77 in the first 15 minutes, reaching 4.87 after 7 hours and stabilizing around 4.54 after 3 days operating in SBR modus. The NoMaB reactor acted as a control reactor fed with flue gas and sewage but without addition of MaB-flocs. It contained 0.192 ± 0.031 g VVS L^{-1} and 0.4 ± 0.1 mg Chl*a* g^{-1} VSS. In this reactor, the effluent pH stabilized around 6.31 ± 0.07. This indicated the good pH buffering capacity of sewage compared to deionised water. In the sewage fed reactors containing MaB-flocs without flue gas ($MaB_{0.0\ L}$) and with 0.6 L h^{-1} flue gas ($MaB_{0.6\ L}$), the Flemish discharge standard for the pH of sewage effluent (6.5 – 9.0; Vlarem II, 1995) was met (Fig. 3.2.c). However, increasing

the flue gas flow rate to 1.2 L h^{-1} led to a pH of 6.28 ± 0.06. This is lower than the discharge standard (Fig. 3.2.c). The in $MaB_{0.0L}$ obtained pH of 7.28 ± 0.07 showed that, the addition of flue gas is not needed to decrease the pH of the MaB-floc reactor to stay within the optimal pH for discharge and biological growth. This can be explained by the low HRT applied, as the pH increased by microalgal photosynthesis during one SBR cycle was smaller than the pH decrease by nitrification and flue gas addition.

The effluent of $MaB_{0.6\ L}$ had a turbidity more than 20 times lower than the influent turbidity (1.6 ± 0.6 FTU compared to 44.2 ± 15.3 FTU, respectively). The decrease of the turbidity in the $MaB_{0.6\ L}$ was significantly higher than the control reactor where no MaB-flocs were added (20.3 ± 2.1 FTU; NoMaB). These results show that MaB-flocs had a positive effect on the decrease of the effluent turbidity.

3.3.2. Flue gas treatment

Our off gas concentrations of NO_x and SO_2 were much lower than the current European emission limit values for coal combustion plants (> 300 MW thermal input) of 200 mg Nm^{-3} NO_x and 150 mg Nm^{-3} SO_x (EU, 2010) (Fig. 3.3.). The MaB-floc reactor supplemented with 0.6 L h^{-1} flue gas ($MaB_{0.6\ L}$) showed the highest removal efficiency of CO_2, NO_X and SO_2: 49 ± 6 % of the CO_2, 87 ± 5 % of the N-NO_x and 99 ± 1 % of the SO_2 was removed (Fig. 3.3.; Table 3.2.). This was obtained under a low PPFD of 100 µmol PAR photons m^{-2} s^{-1}. NO was for a minor part oxidised to NO_2 (Fig. 3.3.), therefore removal efficiencies and removal rates were calculated as N-NO_x on weight basis (Table 3.2.). Increasing the gas flow rate from 0.6 to 1.2 L h^{-1} significantly decreased the CO_2 removal efficiency, but not of NO_x and SO_2 (Table 3.2.). The CO_2 and NO_x removal efficiencies of the MaB-floc reactor were similar compared to previous work with non-flocculent microalgal cultures, but the removal rates were lower (Table 3.2.). This was due to the low flue gas rate applied in our study. Since, a lower flue gas flow rate combined with an equivalent CO_2 concentration of this sparged flue gas and a similar CO_2 removal efficiency leads to a lower CO_2 removal rate. Whereas SO_2 has a high solubility in water (85 g L^{-1} at 25 °C), NO is a gas with a low solubility in water (Jin et al., 2005). Therefore, a chelating agent like Fe(II)EDTA is often used to enhance the NO solubility in the water phase. In this study, no chelators were added. However, they might have been present in the

sewage (for example, EDTA is a common component in shampoo and detergents) or produced by the microalgae, since photosynthetic microorganisms can produce iron siderophores (Mahanesh, 1991) which may act as bio-chelators for NO, similar to Fe(II)EDTA. The high NO_x removal efficiency in the wastewater fed MaB-floc reactors can be attributed to: (1) the use of primary treated sewage, since 50 % of the N-NO_x was already removed in NoMaB compared to 18 % in RH_2O and (2) the presence of MaB-flocs, since $MaB_{0.6\ L}$ showed a N-NO_x removal efficiency (weight based) of 87 % compared to 50 % in NoMaB (Table 2.3.).

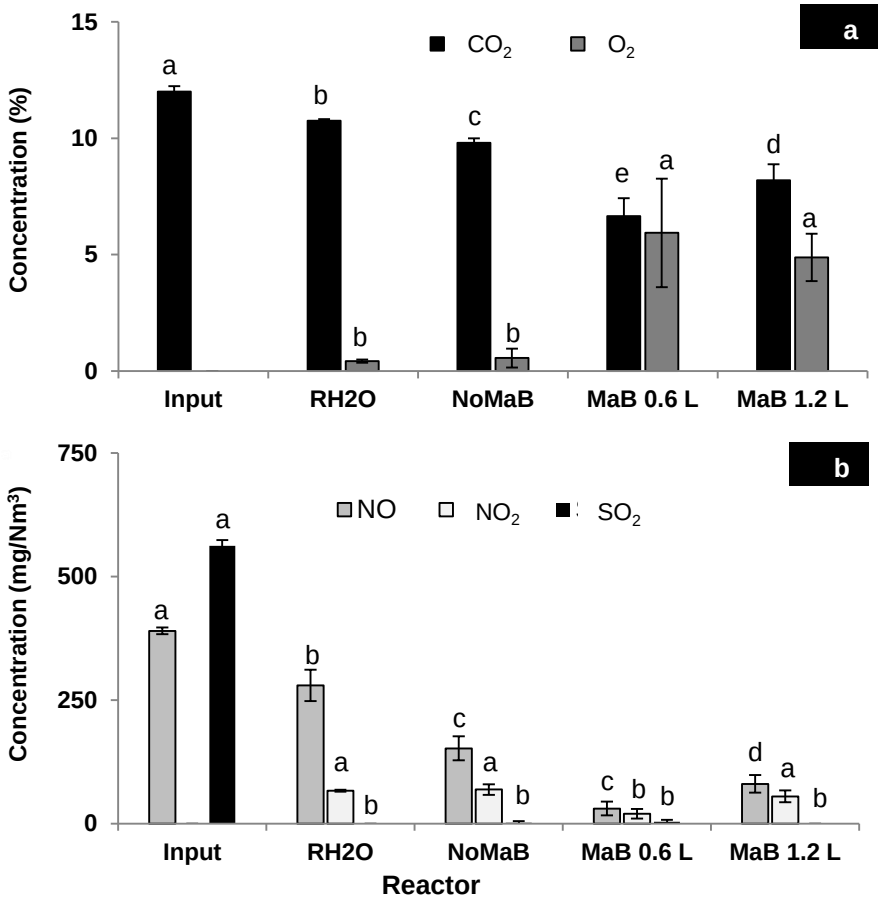


Fig. 3.3. *Flue gas compositions before (input) and after treatment in MaB-floc SBRs supplemented with 0.6 L h^{-1} ($MaB_{0.6L}$) and 1.2 L h^{-1} ($MaB_{1.2L}$) flue gas: CO_2 and O_2 (a) and NO, NO_2 and SO_2 (b)*

The reactors RH_2O (deionized water) and NoMaB (sewage) without MaB-flocs and sparged with 0.6 L h^{-1} flue gas served as controls. The ranges indicated with a different superscript (a-e) within the same pollutant are significantly different from each other according to a Kruskal-Wallis test followed by a Mann-Whitney test with Bonferroni correction ($p < 0.05$).

Table 3.2. *Ingas concentrations, removal efficiency (RE) and removal rate (RR) of CO_2 and NO_x in MaB-floc SBRs compared to previous studies with microalgae*

Species or reactor	**FGFR ($L\ h^{-1}$)**	**CO_2**			**NO_x**			**Reactor volume (L)**	**Reference**
		Conc. in[1] ($g\ CO_2\ Nm^{-3}$)	**RE (%)**	**RR[2] ($mg\ C\text{-}CO_2\ h^{-1}\ L^{-1}$)**	**Conc. in[1] ($mg\ NO_x\ Nm^{-3}$)**	**RE[3] (%)**	**RR[2] ($\mu g\ N\text{-}NO_x\ h^{-1}\ L^{-1}$)**		
Chlorella vulgaris	180	20	60	71	-[4]	-	-	9	Cheng et al., 2006
Chlorella sp.	12	197	20	161	-	-	-	0.8	Chiu et al., 2008
Chlorella sp.	12	39	58	93	-	-	-	0.8	Chiu et al., 2008
Dunaliella tertiolecta	18	-	-	-	402	55	464	4	Nagase et al., 2001
Dunaliella tertiolecta	9	-	-	-	134	65	91	4	Nagase et al., 1997
HSA	180	295	3	434	-	-	-	1	Hsueh et al., 2007
NOA-113	9	295	nd[5]	10	402	46	194	4	Yoshihara et al., 1996
Scenedesmus sp.	18	-	-	-	402	43	1452	1	Jin et al., 2008
$MaB_{1.2\ L}$	1.2	236	38 ± 5^{b}	18.4 ± 2.4^{a}	383	70 ± 5^{b}	35.2 ± 2.4^{a}	4	This study
$MaB_{0.6\ L}$	0.6	236	49 ± 6^{a}	4.7 ± 0.6^{b}	397	87 ± 5^{a}	23.2 ± 1.2^{b}	4	This study
NoMaB	0.6	236	26 ± 1^{c}	2.5 ± 0.1^{c}	383	50 ± 5^{c}	12.5 ± 1.4^{c}	4	This study

[1] Calculated to standard conditions: 273.15 K and 101325 Pa; [2] Removal rates per liter reactor volume; [3] Calculated from sum of N-NO and $N\text{-}NO_2$ in $mg\ Nm^{-3}$; [4] -: not investigated; [5] nd: no data [a,b,c] The ranges indicated with a different superscript within the same column are significantly different from each other according to Kruskal-Wallis test followed by a Mann-Whitney test with Bonferroni correction ($p < 0.05$).

The photosynthetic oxygen present in the off gas (Fig. 3.3.) suggests aerobic conditions in the reactor and therefore anaerobic digestion (loss of carbon as CH_4 and CO_2) and denitrification (loss of nitrogen as N_2 and N_2O) was not expected. Microalgal photosynthesis does not only produce oxygen, but may also diminish the release of CO_2 to the atmosphere, which may alleviate the trend towards global warming. A flue gas rate of 1.2 L h^{-1} led to an increase of 9.18 ± 6.24 mg TIC L^{-1} sewage effluent, indicating that this flue gas flow rate was higher than was needed to compensate the carbon limitation of sewage. From this it can be concluded that in this lab-scale reactor set up, a flue gas rate of 0.6 L h^{-1} was sufficient. In $MaB_{0.6\ L}$, 9.9 ± 6.5 g TIC m^{-3} sewage was removed from the sewage and 63.3 ± 7.8 g CO_2-C m^{-3} sewage was removed from the flue gas. With the current price for carbon credits of 15.30 € per ton CO_2, the latter inorganic carbon removal (from gas phase) would lead to 0.97 € per 1000 m^3 of sewage or only 0.19 % of the current sewage treatment cost of 500 € per 1000 m^3 sewage.

3.3.3. Bioflocculation

Since the goal is lowering the cost of biomass harvesting, it is crucial that MaB-flocs settle fast, and this up to a high density, leaving a clear supernatant. In our experiments, MaB-flocs settled in half an hour up to 23.2 g TSS L^{-1} or 19.2 g VSS L^{-1} being nearly 2 % dry matter biomass (reactor $MaB_{0.6\ L}$; Table 3.3.). This is nearly double than earlier reported densities of 12.5 g TS L^{-1} (Gutzeit et al., 2007) and 13.2 g TSS L^{-1} (Van Den Hende et al., 2011b). Moreover, the biomass settling time was only 30 minutes, being six times lower than reported in sewage and flue gas fed HRAPs (Park and Craggs, 2010b). The effluent turbidity and total organic carbon (TOC) content were low (Table 3.3.). Furthermore, these average TOC levels of 12.0 - 17.7 mg C L^{-1} mean a maximum microorganisms content in the effluent of 24.0 – 35.4 mg L^{-1} (assuming a 50 % organic carbon content of microalgae), and thus, a microalgal bacterial biomass recovery of minimum 97.5 %. This gravity settling led to biomass densities comparable to those of a first concentration step of microalgae harvesting, but did not use electricity or extra materials such as screens. For example, natural filtration and screening with a low vibrating screen filter leaded to 1 - 6 % TSS but uses 0.4 kWh m^{-3}; gravity sedimentation with a lamella separator to 0.5 - 1.5 % TSS uses 0.1 kWh m^{-3}; air flotation to 1 - 6 % TSS uses 2.06 kWh m^{-3} (Uduman et al.,

2010 and references therein). This shows that bioflocculation can lower the biomass harvesting costs by providing a nearly cost-free first harvesting step, as only (a short) time is the prerequisite. The need and choice of a second harvesting step, such as centrifugation, will depend on the application and composition of the biomass.

***Table. 3.3**. Biomass productivity and settling properties of MaB-flocs from sewage fed SBRs supplemented with flue gas at a flow rate of 1.2 L h^{-1} ($MaB_{1.2\ L}$), 0.6 L h^{-1} ($MaB_{0.6\ L}$) and 0.0 L h^{-1} ($MaB_{0.0\ L}$)*

NoMaB served as a control reactor (sewage with 0.6 L h^{-1} flue gas without MaB-flocs)

Reactor	Biomass productivity (g VSS L^{-1} day^{-1})	SVI (ml g^{-1} TSS)[1]	TSS (g L^{-1})	VSS (g L^{-1})	Settled floc density (g VSS L^{-1})[2]	Effluent turbidity (FTU)	Effluent TOC (mg C L^{-1})
$MaB_{1.2\ L}$	0.153±0.039	115–158	1.221±0.080	0.961±0.058	4.9–7.7	11.9±3.0[b]	12±6
$MaB_{0.6\ L}$	0.181±0.122	43–70	1.793±0.443	1.469±0.309	19.2–10.1	1.6±0.6[c]	18±7
$MaB_{0.0\ L}$	0.151±0.111	103–120	1.310±0.304	0.957± 0.226	6.1–6.8	1.1±0.3[c]	13±11
NoMaB	-[3]	-[3]	0.272±0.033[4]	0.192±0.031[4]	-[3]	20.3±2.1[a]	19±7

[1] Range of only 3 measurements; [2] Floc density after 30 min of settling; [3] Not relevant; [4] No Kruskal-Wallis test possible due to small dataset; [a,b,c] The ranges indicated with a different superscript within the same column are significantly different from each other according to Kruskal-Wallis test followed by Mann-Whitney test with Bonferroni correction ($p < 0.05$).

In this study, a consortium of local photosynthetic microorganisms was used, isolated from a pond in the vicinity of a coal fired thermoelectric power plant as a strategy to obtain species with the ability to grow in the presence of the combustion gases (de Morais and Costa, 2007). This inoculum contained predominantly *Chlorella* sp., *Pediastrum* sp., *Phormidium* sp. and *Scenedesmus* sp.. Microscopic observations showed a good incorporation of photosynthetic microorganisms in the flocs (Fig. 3.4.), with *Chlorella* sp. the predominant microalga. This chlorophytic microalga can grow well even in raw wastewater conditions (Pittman et al., 2010). Whereas the addition of inorganic carbon, in the form of bicarbonate, to synthetic wastewater fed MaB-floc reactors led to a decrease of the floc settleability due to the dominance of filamentous *Phormidium* sp. (Van Den Hende et al., 2011b), this effect was not observed by the addition of CO_2 from flue gas to a sewage fed MaB-floc reactor. In all reactors of this study, *Phormidium* species were present but did not grow outside the flocs. Moreover, no increase in abundance of *Phormidium* species was observed by adding flue gas. Also, the addition of flue gas did not significantly decrease the

physiological condition (FC) of microalgae, evaluated by means of the $A664_b$:$A665_a$ ratio (Table 3.4.). A ratio of 1.7 indicates pure chlorophyll *a* (Chl*a*) and a good FC of microalgae; a ratio of 1.0 indicates pure pheophytin*a* (i.e. Chl*a* lacking a central Mg^{2+} ion) and no living microalgae. This suggests that nor 0.0025 vvm nor 0.0050 vmm flue gas was toxic for the photosynthetic microorganisms.

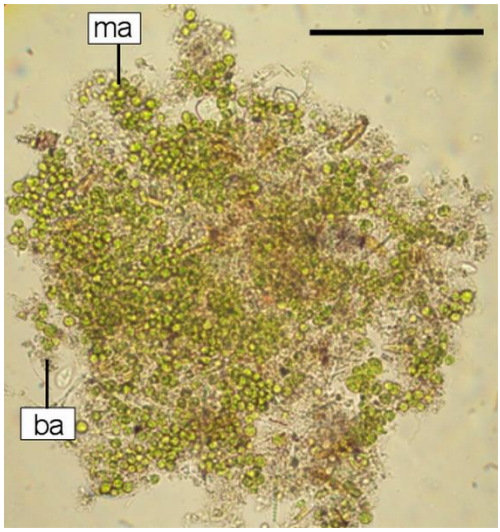


Fig. 3.4. *Light microscopy image (400x) of a MaB-floc from* $MaB_{0.6\ L}$ *at day 10 showing microalgae (ma) and bacteria (ba)*
Depicted scale bar measures 50 µm in length.

To assess the proportion of microalgae and bacteria in an aquatic community, the AI, a standard parameter used in periphyton with values ranging from 50 to 350 (APHA et al., 2003), was determined. The in our study obtained AI for MaB-flocs were low in all reactors (Table 3.4.) indicating an autotrophic dominance in the microalgal bacterial communities. This suggests a high microalgae:bacteria ratio of the biomass. A common method to assess the microalgae content in microalgae bacteria ponds is measuring the Chl*a* content and assuming that the microalgal biomass has a constant Chl*a* content of 1.5 % of the dry weight (Park and Craggs, 2010b). However, the actual Chl*a* content of microalgal cells varies with the microalgal species, cell density and growth conditions (particularly light availability) (Park and Cragss, 2010). Our results (Table 3.4.) confirmed this inaccuracy since in $MaB_{0.6\ L}$ we obtained an average Chl*a* content of 1.87 ± 0.53 % of the dry weight of

MaB-flocs including microalgae and bacteria. This suggests a Chl*a* content of microalgae above 1.87 %. This high value is probably due to the low irradiation level and the high cell density applied. More accurate methods for the determination of microalgae and bacteria are needed, especially in mixed and flocculating biomass samples.

Table 3.4. *Physiological condition ($A664_b$:$A665_a$), autotrophic index (AI) and chlorophyll a (Chla) content of sewage fed MaB-flocs sparged with flue gas at a flow rate of 1.2 L h^{-1} ($MaB_{1.2\ L}$), 0.6 L h^{-1} ($MaB_{0.6\ L}$) and 0.0 L h^{-1} ($MaB_{0.0\ L}$)*
NoMaB served as a control reactor (sewage with 0.6 L h^{-1} flue gas without MaB-flocs)

Reactor	$A664_b$:$A665_a$	AI	Chl*a* (mg g^{-1} VSS)
$MaB_{1.2\ L}$	1.58 ± 0.06	133 ± 35[a]	8.0 ± 2.4[b]
$MaB_{0.6\ L}$	1.54 ± 0.06	62 ± 14[b]	18.7 ± 5.3[a]
$MaB_{0.0\ L}$	1.58 ± 0.02	110 ± 18[a]	9.3 ± 1.6[b]
NoMaB	1.23 ± 0.17[1]	2326 ± 505[1]	0.44 ± 0.10[1]

[1] Kruskal-Wallis test was not possible due to small dataset; [a,b,c] The ranges indicated with a different superscript within the same column are significantly different from each other according to Kruskal-Wallis test followed by Mann-Whitney test with Bonferroni correction ($p < 0.05$).

3.3.4. Biomass production

As sewage treatment is the primary goal, a high microalgal biomass productivity is not the main driver, since biomass is a by-product. Nevertheless, the productivity is intrinsically linked to the microalgal biomass production and it can be assumed that its valorisation is of major importance for the feasibility of this technology.

The average daily productivities of all reactors ranged between 0.151 and 0.181 g VVS L^{-1} reactor day^{-1} or 101 and 121 g VSS m^{-3} wastewater treated. The highest biomass productivity was obtained in $MaB_{0.6\ L}$. This biomass production is comparable to those in other labscale photobioreactors (Kumar et al., 2010). A gastight MaB-floc photobioreactor was used in our experiments to monitor the flue gas treatment. However, MaB-floc reactors on an industrial scale should be high rate algae ponds (HRAPs). Without improvements, the productivity of MaB-flocs in full scale HRAPs is expected to be lower than lab-scale results due to factors such as diurnal and seasonal variations in light and temperature, lower irradiated surface-to-volume ratio, higher but fluctuating photon flux densities and maintenance downtime.

Microalgal productivities measured in both commercial production and wastewater treatment HRAPs range widely from 12 - 40 g m^{-2} day^{-1} or 0.04 - 0.13 g $L^{-1}_{reactor}$ day^{-1} (Park et al., 2011a). The conversion of lab-scale productivities to outdoor conditions in HRAPs is highly dependent on the extrapolation factor applied, which is difficult to precisely assess and therefore sometimes non-realistically equalled to one. Nevertheless, our results seem promising, since, for example, a severe extrapolation factor of 0.3 would still lead to 67 ton ha^{-1} $year^{-1}$ in a 0.40 m deep pond.

3.3.5. Biomass valorisation

Not the utilization of flue gas (Kastanek et al., 2010), but the use of sewage and coagulation with sewage bacteria will hamper its compliment with the EU requirements for food and will limit the valorisation of flue gas and sewage fed MaB-flocs to non-food products. Preferably, the valorisation is a combination of higher value products for the chemical industry such as pigments, low value products such as soil conditioners (N-P-fertilizers, biochar) and recovery of energy (biogas, biodiesel). Algal pigments such as chlorophyll *a*, present in all photosynthetic microorganisms, have commercial value as natural colorants, for example, for dying fabrics and paints (Prasana et al., 2007). The Chl*a* production in our study was highest in $MaB_{0.6\ L}$, being 3.48 mg Chl*a* L^{-1} reactor day^{-1} or 2.26 g Chl*a* m^{-3} sewage treated. Whereas current bulk prices for microalgal pigments for the food industry range around 250 € kg^{-1}, non-food prices are only around 5 € kg^{-1} leading to maximum 11 € for all the Chl*a* from MaB-flocs produced with 1000 m^3 of sewage. This value is around 50 times lower compared to the average treatment cost of sewage (0.5 € m^{-3}).

As a first indication for estimating the valorisation potential of bio-energy from MaB-flocs, a screening was done for lipids. The MaB-flocs contained 14.1 ± 3.3 % w/w (6 measurements) and 23.6 ± 1.1 % w/w (2) of lipids and 11.5 ± 0.3 % w/w (2) and 16.8 ± 1.6 % w/w (2) of neutral lipids, in $MaB_{1.2\ L}$ and $MaB_{0.6\ L,}$ respectively. These concentrations of neutral lipids of MaB-flocs were 1.5 to 2.2 times higher than of activated sludge (Huynh et al., 2010). The highest obtained lipid productivity of 42.7 mg lipids L^{-1} day^{-1} ($MaB_{0.6L}$) seems promising compared to that of lab-scale reactors with pure microalgae cultures at the same light intensity as in this study, ranging from 17.4 – 61.0 mg lipids L^{-1} day^{-1} (Rodolfi et al., 2008). The assessed MaB-floc productivity of 67 ton ha^{-1} $year^{-1}$ (in section 3.3.4.) could lead to 16 ton of lipids

ha $^{-1}$ year^{-1} and 2.6 ton neutral lipids ha^{-1} year^{-1}, indicating the potential of MaB-flocs for bio-energy production.

3.4. Conclusions

In general, the MaB-floc reactor for treatment of primary sewage supplemented with flue gas from coal burning at a gas flow rate of 0.6 L h^{-1} (0.0025 vvm) showed the best reactor performance, compared to 0.0 and 1.2 L h^{-1}. Moreover, European discharge standards for sewage (N, P) and offgas (NO_x, SO_x) were met with a low hydraulic retention time of only 0.67 days. This successful sewage and flue gas treatment, together with their settling up to 19 g VSS L^{-1}, make microalgal bacterial flocs (MaB-flocs) a promising alternative for conventional sewage treatment. However, data matching to outdoor high rate algae ponds and valorisation of the biomass has yet to be investigated, before the stage is set for the industrial implementation of MaB-floc ponds. Furthermore, flue gas contains next to CO_2, NO_x and SO_x also other chemical compounds. The interaction of all these compounds with microalgae needs further study.

3.5. Acknowledgements

This work was supported by a Ph.D. grant from University College West-Flanders and UGent. The authors thank E. Ryckebosch (KULeuven) for the lipid analyses; G. Bellens and X.Vanhoutte (Howest) for their technical assistance; G. Van de Velde (UGent) for her lab assistance; G. Maes and B. Vanslambrouck (Howest) for their useful suggestions; H. Auvinen, C. Boeckaert and B. Decostere (Howest) for critically reading the manuscript.

Everything has beauty,

but not everyone sees it.

(Conficius, 551BC-479BC)

Picture on previous page:

Flue gas emission at a coal burning power plant nearby Xi'An in China.

CHAPTER 4

Flue gas compounds and microalgae: (bio-)chemical interactions leading to biotechnological opportunities

Abstract

Flue gases are a resource yet to be fully utilised in microalgal biotechnology, not only to moderate the anthropogenic effects on our climate, but also to steer microalgal resource management towards innovative applications of microalgal biomass compounds. These gases, both untreated and treated into current discharge standards, contain CO_2, N_2, H_2O, O_2, NO_x, SO_x, C_xH_y, CO, particulate matter, halogen acids and heavy metals. To better steer and engineer flue gas-fed microalgal cultures, all these compounds need to be considered. Therefore, here, this chapter reviews (i) the chemical composition and treatment technologies of flue gas, (ii) the uptake pathways and the removal of different flue gas compounds in microalgae reactors, and (iii) the tolerance and effects on microalgae of all flue gas compounds. By emphasising the interactions between microalgae and flue gas compounds, new pathways for microalgal biomass valorisation such as enzyme production for environmental technology, novel biogas production and biosequestration of minerals are envisaged. Furthermore, fundamental and applied research niches that merit further investigation are highlighted.

Chapter redrafted after:

Van Den Hende, S., Vervaeren, H., Boon, N., 2012. Flue gas compounds and microalgae: (bio-)chemical interactions leading to biotechnological opportunities. Biotechnol. Adv. 30, 1405-1424.

4.1. Introduction

Both global warming concern and sustainable resource management are of rapidly growing importance in our societies and present an opportunity for microalgae biotechnology. Whether the main application of microalgae is environmental technology or the production of bio-energy, feed, food and fine chemicals (Becker, 2008), sustainable microalgal technology needs cost-effective resource management. Carbon is a key resource for successful microalgal production as it is the main element of microalgae (36 to 65 % of the dry matter; Chae et al., 2006; Sydney et al., 2010).

The diffusion of CO_2 from the atmosphere into a microalgal culture is not efficient enough to obtain high biomass productivity due to the low CO_2 content of air (McGinn et al., 2011) and the high surface tension of water (Becker, 2008). Adding inorganic carbon as bicarbonate salts or as compressed CO_2 gas involves a relatively large cost. Especially since at least 1.3 to 2.4 kg CO_2 is required per kg of dry algae (based on 36 to 65 % C content of dry algae) and current prices for commercially delivered CO_2 are in the range of 30 to 45 € per ton of CO_2 (Becker, 2008). With CO_2 uptake efficiencies ranging between 20 and 70 % (Table 4.1.), this would mean a total CO_2 cost ranging between 0.06 and 0.54 € per kg of dry algae. According to Molina Grima et al. (2003), the supply of compressed CO_2 can be up to 41 % of the total costs of raw material. Moreover, supplying concentrated CO_2 in undeep ponds leads to significant losses of CO_2 to the atmosphere (Richmond, 2004). In this regard, the direct use of flue gas containing CO_2 is economically and environmentally a better option than compressed CO_2, especially when combustion plants are close to the location of microalgal cultivation.

Besides CO_2, flue gas can contain up to 142 different compounds (Simoneit et al., 2001) such as H_2O, O_2, N_2, nitrogen oxides (NO_x), sulphur oxides (SO_x), unburned carbohydrates (C_xH_y), CO, heavy metals, halogen acids and particulate matter (PM). Flue gas treated to current emission threshold values still contains non-neglectable concentrations of several compounds (Table 4.1.), some of which can be toxic for microalgae (e.g., SO_2; Lee et al., 2000a), while others can be metabolised by microalgae (e.g., NO; Nagase et al., 2001).

Table 4.1. *Chemical composition of raw flue gas (compounds and indicative ranges of concentrations), European emission limits, factors influencing their concentration, and post-combustion treatment technologies*

Com-pound	Unit	Concen-tration in flue gas	European emission limits[a, 1]	Factors influencing concentration[b]	Post-combustion treatment technologies
N_2	%	77[2] 72-74[3]	-[c]	T, λ[15]	-
CO_2	%	4.8-26.9[4] 7.5[2] 9.5-16.5[5] 5.0-15.0[6] 10.0-3.0[7] 5.5-7.5[8]	-	F[4] T, λ[15]	Absorption: alkaline amine scrubbing, cyclic carbonation/decarbonation with a solid metal oxide, seloxol, rectisol, fluor, purisol process Adsorption: carbon based sorbents, amine functionalized sorbents, lime carbonation/calcinations, pressure swing, temperature swing, electrical swing Cryogenics: anti-sublimation and Integrating Tri-Reforming Power Plant Membranes filtration: polymeric, ceramic, facilitated transport, carbon molecular sieve, membrane contactors[11, 17, 18]
H_2O	%	9.0-13.8[3]	-	F[4] T, λ[15]	-
O_2	%	2.0-6.5[5] 0.7-15.0[3] 3-8 [9], 8-9.5[8]	-	F[4] λ[15]	-
NO	mg Nm^{-3}	59-1500[4] 50-600[10]	50-500 (NO and NO_2 as NO_2)	F[4] T, λ[15] Residence time[16]	Selective catalytic reduction, selective non-catalytic reduction (with urea, NH_3 or cyanuric acid)[19, 20] Adsorption: zeolites, activated carbon, fly ash[19, 20, 21] Absorption: alkaline solutions, strong oxidizing reagents, iron chelates[19, 20]
NO_2	mg Nm^{-3}	2-75 (5 % of NO_2)[11]	50-500 (NO and NO_2 as NO_2)	F [4], T, λ[5]	Hollow fibres and membrane bioreactors[22] Electron beam radiation, non thermal plasma or photocatalytic oxidation[20] Bioreactors: BioDeNox, fungi reactors, microalgae reactors[19]

SO_2	mg Nm^{-3}	0-800[4] 20-1400[10]	5-1000 (SO_2)	F[4] T[15]	Once-trough wet: limestone forced oxidation, limestone inhibited oxidation, jet bubbling reactor, magnesium enhanced lime, dual alkali, seawater scrubbing[23] Once-trough dry: lime spray drying, furnace sorbent injection, LIFAC, economizer sorbent injection, duct sorbent injection, duct spray drying, circulating fluidized bed, Hypas sorbent injection, fly ash-$Ca(OH)_2$ mixture, coal ash[23] Regenerable wet: sodium sulfite, magnesium oxide, sodium carbonate, amine process, citrate process, liquid ammonia[23] Regenerable dry: activated carbon, alkalized alumina, calcium oxide, COBRA process, OSCAR process[23] Biological: direct biofiltration, indirect two-stage with inorganic iron oxidation, bacterial reduction with sorbent regeneration, two-step bacterial reduction, chemical alkaline sorption followed by biological reduction to sulfur[23]
SO_3	mg Nm^{-3}	0-32 (0-4% of SO_2)[4]	-	F[4] T[15]	
CO	mg Nm^{-3}	2.5-5[4] 100-11250[3] 50-100[10]	50-150	F[4] T, λ[15]	CO boiler[10] Catalytic methanation, palladium-based membrane purification, catalytic selective CO oxidation[24]
Particulate matter	mg Nm^{-3}	120-800[12] 2000-15000[14]	5-150 (dust)	F[4] T, λ[15]	Cyclones, multicyclones, wet scrubbers, electrostatic precipitators combined with flue gas conditioning with injection of chemical additives or water, fabric filters or bag filters[10]
C_xH_y	mg Nm^{-3}	0.008-0.4 %[3] < 10^{-3} mg dioxins and furans per kg flue gas [3]	10-20 (total organic carbon) 0.0001 (dioxins and furans)	F[4] T, λ[15]	VOC removal in general: adsorption, incineration, absorption, condensation, membrane, photochemical oxidation, biological treatment[4]

Heavy metals	mg Nm^{-3}	2.2 (sum of all metals)[4] 0.00008-0.0518 (single metal)[13]	0.05 (Cd and Tl) 0.05 (Hg) 0.5 (Total of Sb, As, Pb, Cr, Co, Cu, Mn, Ni and V)	F, T, metal content of ash, fuel chloride content, flow pattern leading to solids drop-out[4]	Partly removed by particulate control systems: cyclones, wet scrubbers, electrostatic precipitators, fabric filters[10] Adsorption to fly ash[2], carbon[25]
Chlorine and its compounds	mg Nm^{-3}	0-1400 (HF)[4] 250-2000[14]	10 (HCl)	F[4]	Absorption by ash, spray drying absorber, wet absorption, dry absorption[4]
Fluorine and its compounds	mg Nm^{-3}	0.5-5 (HF)[14]	1-2 (HF)	F[4]	Absorption by ash, spray drying absorber, wet absorption, dry absorption[4]

[a] Calculated at 273.15 K and 101.3 kPa after correction for water vapour and standardized to a fixed reference O_2 concentration (3-15 %) depending on fuel type; [b] λ: excess air value; F: fuel type; T: burning temperature; [c] - : no emission threshold values or treatment technologies applicable; [d] No limit, but concentration affects legislation limits, by comparing it to reference O_2, to prevent dilution of flue gas; [1] EU, 2010; [2] de Godos, 2010; [3] Merker et al., 2006; [4] Niessen et al., 2002; [5] Lee et al., 2000a; [6] Kanniche et al., 2010 ; [7] Lee and Lee, 2003; [8] Doucha et al., 2005; [9] Zhang et al., 2008; [10]Trozzi et al., 2010; [11] Wang et al., 2008a; [12] Shantakumar, 2008; [13] Reddy et al., 2005; [14] WHO, 1988; [15] Saxena and Thomas, 1995; [16] Van Loo and Koppejan, 2008; [17] Olajire, 2010; [18] Stolten and Scherer, 2011; [19] Jin et al., 2005; [20] Skalska et al., 2010;[21]Ahmaruzzaman, 2009; [22] Min et al., 2004; [23] Pandey et al., 2005; [24] Avgouropoulos and Ioannides, 2003; [25] Liu and Liu, 2011.

To date, most research on flue gas in microalgal cultures has focused on CO_2 (Ho et al., 2011; Jacob-Lopes et al., 2010; Lee and Lee, 2003). If we are to better engineer flue gas-fed microalgal cultures, all flue gas compounds and their interactions with microalgae need to be assessed. Therefore, this review gives an overview of the compounds present in flue gas together with their discharge standards and post-combustion treatment technologies. Current knowledge on the effects of all flue gas compounds on microalgae and vice versa of microalgae on the removal of these compounds is reviewed. This review does not include the interactions of microalgae and flue gas compounds with other microorganisms which can be present in microalgal cultures. For CO_2 as well as for NO_x and SO_x, uptake pathways by, tolerance to, effects on and removal capabilities of microalgae are described in detail. Furthermore, this review identifies knowledge gaps and unexplored pathways, and highlights biotechnological opportunities to use flue gas in microalgae biotechnology.

4.2. Flue gas compounds and current treatment technologies

Flue gas from combustion processes contains several compounds (Table 4.1.): (1) those not restricted by legislation: N_2, O_2 and H_2O; and (2) those restricted by legislation and removed in an additional gas treatment step: CO_2, NO_x, SO_x, CO, C_xH_y, PM, heavy metals, chlorine and fluorine and their compounds. To obtain discharge standards of the latter compounds, several treatment technologies can be employed (Table 4.1.).

4.2.1. Compounds not restricted by legislation

Since most combustion processes use air (except oxy-combustion, where pure oxygen is used), flue gases are generated that mainly contain N_2. This N_2 gas does not have a major part in the combustion process, but a small quantity (< 0.1 %) of it can be oxidised to hazardous nitrogen oxides (NO_x) (Merker et al., 2006). Since an excess of O_2 is needed for a good combustion process, O_2 is present in flue gas at relatively high concentrations (Table 4.1.). Water vapour is also present in flue gas. Despite being a greenhouse gas, there are currently no legislation standards for water vapour in flue gas because of its low atmospheric residence time of about nine days (Schmidt et al., 2010).

4.2.2. Compounds restricted by legislation

Carbon dioxide is formed during the combustion of fuel carbon and its concentration in flue gas mainly depends on the fuel type. For example, combustion gasses of fossil fuel power plants contain 5 to 6 %v CO_2 for natural gas burning and 10 to 15 %v for coal burning (USDOE, 2010). In oxy-combustion processes, CO_2 concentrations in flue gas are obtained of up to 75 %v in wet flue gas and 90 %v in dry flue gas (Kanniche et al., 2010). CO_2 is a greenhouse gas with an atmospheric lifetime of 50 to 200 years (Hamitt et al., 1996) and hence, contributes largely to global warming.

Flue gas contains different NO_x species. Nitric oxide (NO) and nitrogen dioxide (NO_2) are the major NO_x species present, whereas nitrous oxide (N_2O), dinitrogen dioxide (N_2O_2), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4) and nitrogen trioxide (NO_3) are not encountered in significant amounts in flue gas (Skalska et al., 2010; Van Loo and Koppejan, 2008). NO can be formed in four different ways in combustion processes: (1) thermal NO can be formed at high temperatures according to the Zeldovich mechanisms from atmospheric nitrogen; (2) prompt NO is already developed in the flame front via the Fenimore mechanism from air nitrogen; (3) fuel NO can be produced by nitrogen portions in the fuel; and (4) NO can be formed via N_2O (Merker et al., 2006). In general, only a small part of the formed NO (e.g., 5 %v of NO; Wang et al., 2008b) is oxidised to NO_2 before the gas leaves the stack.

NO and NO_2 are NO_x of environmental concern, both being major contributors of photochemical smog, acid rain formation and trophospheric ozone formation in urban air (Kurvits and Marta, 1998). NO and NO_2 are not greenhouse gases, but can indirectly affect earth's radiative balance by catalysing trophospheric O_3 formation (Houghton et al., 2001). Furthermore, NO_x participates in eutrophication and ozone removal from the stratosphere, resulting in increased ultraviolet radiation reaching the earth's surface (Johnston, 1992).

Sulphur oxides (SO_x) are released when sulphur, hydrogen sulphides or organosulphur compounds are burned. The proportioning of sulphur between the dioxide (SO_2) and trioxide (SO_3) forms of SO_x depends on the chemistry of sulphur in the fuel, the time sequence of temperature, the composition of flue gas, and the presence and absence of catalytic ash material, but generally only 2 to 4 % of the

sulphur appears as trioxide (Niessen, 2002). They contribute directly to acid rain formation and the degradation of the ozone layer (Niessen, 2002).

In uncomplete combustion, due to a lack of air or a low temperature in an extremely lean mixture, flue gas contains CO and a complex mixture of unburned hydrocarbons (C_xH_y) like methane (CH_4) and butane (C_4H_{10}) (Trozzi et al., 2010). Generally, C_xH_y are divided into aromatics (including polycyclic aromatic hydrocarbons (PAHs), dioxins and furans), alkanes, alkenes, ketones and aldehydes (Merker et al., 2006). C_xH_y are to a large extent responsible for global warming and may have a toxic, carcinogenic, mutagenic and/or teratogenic effect (Niessen, 2002). An optimisation of the burning process can lower the emission of C_xH_y in the stack (Niessen, 2002).

Emissions of particulate matter from burning processes originate from several sources. Aerosols (particles with a diameter less than 1 µm) are a result of reactions between K or Na and Cl or S released during combustion and subsequent formation of submicron particles via nucleation and condensation. Fly ashes (particles with a diameter larger than 1 µm) result from entrainment of ash and fuel particles from the fuel bed (Van Loo and Koppejan, 2008). Soot is developed when PAHs develop into larger formations with a diameter ranging from 10 to 150 nm (Merker et al., 2006).

Most heavy metals present in flue gas are normally released as oxides or chlorides in association with particulates (Trozzi et al., 2010). In general, the less volatile heavy metals (e.g., Cd, Co, Cu, Fe, Zn) are likely to be associated with fine particulate matter as well as bottom ash and fly ash, whereas Hg is volatilised and emitted fully in the vapour phase (Niessen, 2002).

Combustion processes also result in the emissions of chlorine and fluorine, primarily in the form of hydrogen chloride (HCl) and hydrogen fluoride (HF). These halogen acids lead to acid rain and corrosion of ferrous metals (Niessen, 2002). Lower amounts of chlorine and fluorine gas are also emitted. The concentration of halogen acids in flue gas mainly depends on the quantity of halogen in the fuel. For example, coal contains 0.01 to 0.5 %w chlorine, which forms hydrochloric acid during combustion (Evans et al., 2011).

4.2.3. Post-combustion treatment of flue gas before injection in microalgae reactors

The concentration of these restricted compounds in flue gas does not only depend on the fuel type, but also on the combustor system including the fractional excess air value (λ) and the combustion temperature (Trozzi, 2010). For certain flue gas sources the concentrations of some compounds, such as. mercury and cadmium, hydrogen fluorides and chlorides, in the untreated gas are neglectable (Table 4.2.). This implies that certain sources of raw flue gas are more suitable for microalgal cultures than others. For example, in flue gas from natural gas burning, both SOx and heavy metals are neglectable. Since the 1970s, the discharge of flue gas containing high concentrations of pollutants has been controlled and regulated (Mochida et al., 2000). The therefore needed control techniques may be classified into three categories: fuel treatment/substitution, combustion modification and post-combustion control. A wide range of physical, chemical and biological post-combustion treatments exist (Table 4.1.); all having their advantages and disadvantages.

Table 4.2. *Flue gas compounds with potential to be a key category for a certain flue gas source (adapted from Trozzi, 2010)*

Source	**Substance**							
	CO_2, CO	**NO_x**	**SO_x**	**PM**	**C_xH_y**	**Hg, Cd**	**Metals and their compounds[a]**	**Chloride, fluoride**
Boilers and furnaces	x	x	x	x	x	x	x	x
Gas turbine	x	x	(x)[b]	x	x	-	-	-
Compressed injection engine	x	x	x	x	x	-	x	x
Refinery activities	x	x	x	x	x	-	x	x
Coke ovens	x	x	x	x	x	x	x	x

[a] Other than Hg, Cd; [b] Not applicable if natural gas.

Chemical and/or physical CO_2 treatment technologies are relatively costly (22 to 36 € per ton of C; Wang, 2008a) and energy-consuming, so that the CO_2 mitigation benefits become marginal (Skjanes et al., 2007). Moreover, they often have

disposal problems for waste absorbents and captured CO_2 (Wang et al., 2008a). Therefore, biological methods, such as microalgal treatment, have gained a lot of attention lately. Microalgal CO_2 fixation is difficult to compare with Carbon Capture and Storage (CCS) technology, since microalgal CO_2 fixation can be temporary if the microalgal biomass is oxidized to CO_2.

NO_x and SO_x can be treated separately or simultaneously. Whereas conventional NO_x treatments are expensive and produce secondary wastes often requiring further treatment, biological denox methods are still in their experimental phase and merit further studies (Jin et al., 2005). Examples of combined treatment systems are the DeSoNox or SNOX processes, where a catalytic reduction of NO_x is combined with a catalytic oxidation of SO_2 (Trozzi et al., 2010). Physico-chemical desox methods have also some disadvantages: high capital and energy cost, high regeneration cost of sulphates and oxides (if catalysts are used), low removal of SO_3 (50 %), high water usage, non-neglectable cost of limestone, large size of scrubbers, and increased formation of acid mist (in case of wet desulphurisation) (Mochida et al., 2000). Worldwide, wet desulphurisation with a limestone-forced oxidation process is the most commonly used (74 %; Pandey et al., 2005), especially where there is a market demand for the gypsum byproduct and cheap availability of water and sorbents (Mochida et al., 2000). A portion of the chlorine and fluorine in the fuel may be absorbed onto fly ash or bottom ash. Both HCl and HF are water-soluble and are readily controlled by acid gas scrubbing systems together with other acid gases such as SO_2 (Niessen, 2002). Among the dust and heavy metal removal technologies (Table 4.1.), electrostatic precipitators (ESP) are the most effective, especially when they are combined with flue gas conditioning (Shanthakumar, 2008).

In this study, several factors are distinguished which affect the need for and choice of conditioning of flue gas prior to its use in microalgae reactors:

- composition of the raw flue gas, which depends on combustion conditions: the fuel type being burned, the temperature and the fractional excess air value (Table 4.1.);
- toxicity to the aimed microalgae of the flue gas compounds and consequently decreased pH of the reactor liquor (and thus, also the buffer capacity of the medium);

- size of the combustor and discharge standard: if not all flue gas can be injected into the microalgae reactor, gas cleaning is obliged by legislation (for large boilers);
- local legislation: discharge limits of offgas and effluent, methods to assess the amount of CO_2 , NO_x and SO_x credits, and allowance to use open ponds as gas treatment vary among countries;
- composition of wastewater/medium used (pH, C, N, S and metal content);
- economics: the costs of the post-combustion treatment (and intertwined with this, the size of the combustor);
- valorisation of microalgal biomass and its concomitant legislation limits.

A common flue gas post-combustion treatment system of large combustion plants, such as coal power plants, consist of an ESP, DeNox and DeSox step often combined with a heat exchanger (Fig. 4.1.). An additional heat exchanger and condensor may be needed to precede the injection of the flue gas into the microalgae reactor, particularly to avoid corrosion of the fan and lower the flue gas temperature. The flue gas flow rate and heat exchanger should be steared by a programmable logic controller (PLC) in relation to the pH and temperature of the microalgal pond. Where low-efficiency ESPs are still used, an extra ash collector may be required, as applied in flue gas fed microalgal ponds of Seambiotic Inc. (Ben-Amotz, 2009).

Globally, several algae pilot reactors with injection of flue gas have been and are being constructed (Aquafuels, 2011). However, to date, only few examples exist of the industrial application of the use of flue gas in microalgal cultures. Israel Electric Company and Seambiotic Ltd inject flue gas into open ponds with *Nannochloropsis* cultures (Christenson and Sims, 2011). In Hawaii, Cyanotech Corporation uses a patented system to provide flue gas to *Arthropspira* sp. cultures and to recover the heat of the fossil fuel engine to dry the cyanobacteria (Cyanotech Corporation, 1997). Several other systems where flue gas scrubbing is combined with microalgal cultures have been patented. For example, Der and Shang (2001) patented a system where a cascading algae-laden water film on the inside of a stack wall and on surfaces of an optional internally mounted vortex generator entrains small particles and CO_2 from flue gas. This stack can also be used as a photobioreactor. Another example is the wet gas scrubbing of Jennes et al. (2011), where aqueous absorbent solution flows in circuit with the microalgae reactor.

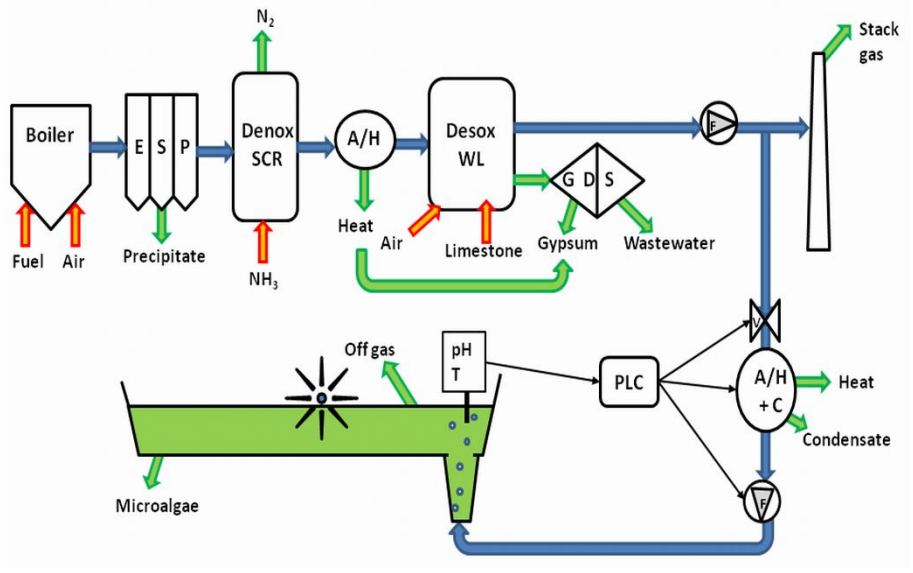


Figure 4.1. *Example of a process flow chart for treating flue gas from a coal power plant combined with injection into a high rate algae pond*
ESP: electrostatic precipitator; SCR: selective catalytic reduction; A/H: air heater; WL: wet limestone gypsum process; GDS: gypsum dewatering system; F: fan; V: valve; A/H: heat exchanger and water condensation; PLC: programmable logic controller.

4.3. Interaction of flue gas compounds and microalgae

To provide insights into the interaction between flue gas compounds and microalgae, an overview of the current knowledge on the solubility, uptake pathways, tolerance to of flue gas compounds on microalgae is given. An overview of the effects of flue gas compounds on microalgae and vice-versa of the effect of microalgae on the compound removal from the gas phase are discussed and presented in Table 4.3.

In this review, the term microalgae covers all unicellular and simple multicellular microorganisms, including eukaryotic microalgae and prokaryotic cyanobacteria, performing oxygenic photosynthesis via:

$$6\ CO_2 + 6\ H_2O + \text{minimum 8 photons} \rightarrow C_6H_{12}O_6 + 6\ O_2 + \text{waste heat} \qquad \text{(E. 4.1)}$$

Table 4.3. *Reported positive (+) and negative (-) effects of flue gas compounds on microalgae and vice-versa*

Com-pound	Effects of flue gas compound on microalgae	Effects of microalgae on compound removal from the gas phase[a]
N_2	+ Nitrogen source for cyanobacteria[1] + Lowers concentration of other compounds in flue gas[2]	+ Uptake by nitrogen fixing cyanobacteria[1]
CO_2	+ Carbon source: component reduced in photosynthesis[1] + Component stored via CCM and calcification[3] + CO_2:O_2 ratio changes affinity for rubisco[4] + Counteracts pH increase due to microalgal growth[5] ± Changes the biochemical composition of microalgae: C-content[6], N-content[7], lipids and fatty acids including EPA and DHA[6, 8], intracellular polysaccharides[8], nitrate reductase[9], chl*a*:chl*b* ratio[9], production of high value biomolecules[10], carbonic anhydrase[11], lower affinity to CO_2 and higher photosynthetic sensitivity to O_2[12] - Lowers productivity at high loadings[13] - Toxical if leading to strong acidification of culture[14] - High concentrations inhibit efficiency of photosystem II[15]	+ Removal via photosynthesis and calcification[1] + Enhancement of CO_2 hydrolysis by production of external carbonic anhydrases[12, 27] - Loss of CO_2 uptake by production of extracellular VOCs by microalgae[28] - Decrease of CO_2 solubility by increase of temperature via photosynthesis[29] - Decrease of CO_2 solubility by increase of liquid surface tension[28] - Loss of CO_2 uptake by photorespiration[1]
H_2O	+ Used as electron donor in photosynthesis[1] + Needed to have an aquatic environment and ionization of compounds[1]	+ Microalgal uptake via photosynthesis[1]
O_2	+ Needed for photorespiration as electron acceptor[1] - May form toxic oxygen radicals damaging cell membranes[16] - Inhibits CO_2 uptake by competing for Rubisco[17]	+ Removal via (photo)respiration[1] + Supersaturation of O_2 in microalgal cultures[17] - Production of O_2 via photosynthesis[1] - Decrease of O_2 solubility by increase of temperature via photosynthesis[29] - Increase of surface tension due to metabolite production[27]

NO_x	+ Nitrogen source[18] + Counteracts pH increase due to algal growth[19] - May be toxic to microalgal cells[20]	+ Removal via uptake[18] + Enhancing NO dissolvement via production of iron siderophores as biochelators?[30] + Preventing oxidation and preventing inactivation of NO chelating agent Fe(II)EDTA[31] - Microalgal production of NO when fed with nitrate[32] - Decrease solvability by increasing surface tension due to production of metabolites[28]
SO_x	+ Sulfur source: if oxidized to sulfate, taken up and metabolized by cells[5] - May be toxic due to bisulfite formation[21] - May be toxic due to pH decrease[22]	+ Uptake by microalgae after oxidation to sulphate[33] - Loss of sulfur due to production of volatile sulfur compounds[1]
CO	+ Carbon source: use by microalgae after bacterial oxidation[23]	+ Uptake by microalgae after microbial oxidation to CO_2[23]
C_xH_y	+ Carbon source: use by microalgae after bacterial oxidation[24]	+ Production of C_xH_y by microalga[34]
Heavy metals	+ Needed trace elements[5] ± Influence the algal metabolism and activity of carbonic anhydrase[25] - High concentrations can be toxic[27]	+ Removal via microalgal assimilation and adsorption[1] + Removal via precipitation due to microalgal induced pH increase[17]

[a] +: Enhanced removal; -: Addition, so decreased removal; [1] Graham and Wilcox, 2002; [2] Merker, 2006; [3] Price et al., 2008; [4] Atomi, 2002; [5] Becker, 2008; [6] Tang et al., 2011; [7] Larsson et al., 1985; [8] Ishida, 2000; [9] Xia and Gao, 2005; [10] Miyasaka et al., 1998; [11] Chinnasamy et al., 2009; [12] Yang and Gao, 2003; [13] Watanabe et al., 1992; [14] Fallowski and Raven, 2007; [15] Xu, 2004; [16] Pulz et al., 2001; [17] Richmond, 2004; [18] Nagase, 2001; [19] EPA, 1999; [20] Radmann and Costa, 2008; [21] Bake et al., 1983; [22] Yang et al., 2004; [23] King, 2001; [24] van der Ha et al., 2011; [25] Wang et al., 2005; [26] Douskova et al., 2009; [27] Colett et al., 2011; [28] Grima et al., 1993; [29] Liu et al., 2011; [30] Van Den Hende et al., 2011a; [31] Santiago et al., 2010; [32] Sakihama et al., 2001; [33] Giordano et al., 2005b; [34] Schirmer et al., 2010.

4.3.1. Carbon dioxide

4.3.1.1. Solubility of CO_2 in aqueous solutions

According to the two-film theory, mass transfer of CO_2 from the gas phase to the cell phase occurs through sequential stages, but is mainly determined by the gas-liquid stage (Markle, 1977). The CO_2 mass transfer rate (N_{CO2}) is approximately given by:

$$N_{CO2} = k_L \alpha (C_{CO2L}^* - C_{CO2L}) \quad \text{(E. 4.2.)}$$

with k_L the liquid-phase mass transfer coefficient, α the specific area available for mass transfer, C_{CO2L}^* the CO_2 concentration in the liquor that equilibrates its actual partial pressure on the gas side, and C_{CO2L} the actual CO_2 concentration in the liquor (Markle, 1977). Several methods have been proposed for enhancing the N_{CO2} by increasing α and/or k_L, for example, microporous hollow-fibre membranes, air-lift bubble columns, stirring, gas injection methods and gas recirculation (Jacob-Lopes et al., 2010). Compared to O_2, the gas-liquid transfer for CO_2 from flue gas is relatively fast, due to its relatively high solubility (1.496 g CO_2 L^{-1} in water at 25 °C and 1 atm, i.e., over 100 times higher than that of O_2). CO_2 solubility is pH dependent, increases with pressure and decreases with increasing salt concentration and increasing temperature (Liu et al., 2011). Thus, to enhance the solubility of CO_2, the medium can be artificially cooled (Satyanarayana et al., 2011).

When CO_2 dissolves in an aqueous medium, it reacts through a set of chemical equilibriums. At a pH lower than 8, the main pathway is direct hydration (Housecroft and Sharpe, 2005; Stumm and Morgan, 1981; with pK at 25 °C; 1 atm):

$$CO_{2(g)} \text{ <-> } CO_{2(aq)} \quad (pK = 1.468) \quad \text{(E. 4.3.)}$$

$$CO_{2(aq)} + H_2O \text{ <-> } H_2CO_3^* \quad (pK = 2.840) \quad \text{(E. 4.4.)}$$

$$H_2CO_3^* \text{ <-> } HCO_3^-{}_{(aq)} + H^+ \quad (pK = 6.352) \quad \text{(E. 4.5.)}$$

$$HCO_3^-{}_{(aq)} \text{ <-> } CO_3^{2-}{}_{(aq)} + H^+ \quad (pK = 10.329) \quad \text{(E. 4.6.)}$$

with $H_2CO_3^* = CO_{2(aq)} + H_2CO_{3(aq)}$, since these species are nearly indistinguishable. The hydration of CO_2 (reaction E. 4.4.) is slow, whereas the dissociation of carbonic acid (reactions E. 4.5. and E. 4.6.) is so fast that H_2CO_3, HCO_3^- and CO_3^{2-} are in equilibrium (Dreybrodt et al., 1996). At a pH above 10, the main pathway is by the attack of hydroxide ions (Housecroft and Sharpe, 2005):

$$CO_{2(aq)} + OH^-{}_{(aq)} \text{ <-> } HCO_3^-{}_{(aq)} \quad \text{(E. 4.7.)}$$

$$HCO_3^-{}_{(aq)} + OH^-{}_{(aq)} \text{ <-> } CO_3^{2-}{}_{(aq)} + H_2O \quad \text{(E. 4.8.)}$$

Therefore, in aqueous environments with a pH between 6.352 and 10.329 (the most common pH of microalgal cultures), bicarbonate is the dominant carbonate species.

4.3.1.2. Uptake and metabolization pathways of inorganic carbon by microalgae

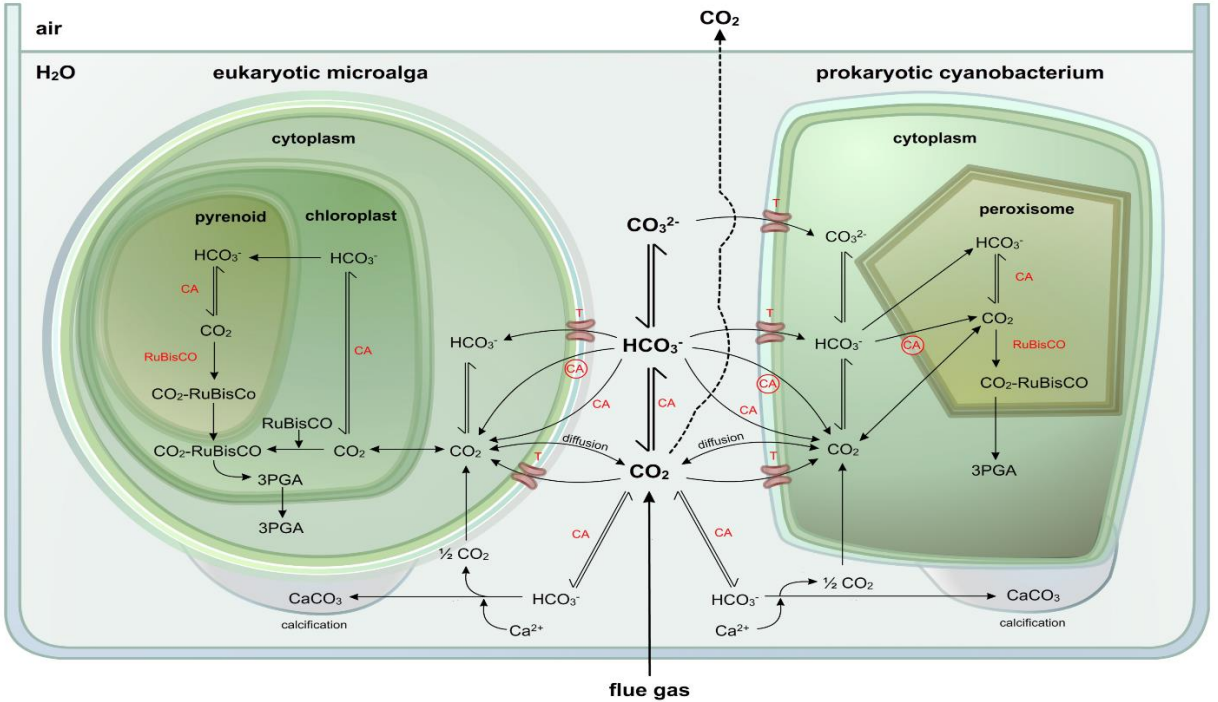
Carbon is the major element of microalgae. The carbon content of microalgae can range between 36 and 65% (Table 4.4.).

Table 4.4. *Carbon content of several microalgal species*

Species	Carbon content (%w)	Reference
Botrycoccus braunii SAG-30.81	58	Sydney et al., 2010
Chlorella pyrenoidosa SJTU-2	49-51	Tang et al., 2011
Chlorella sp. UK001	54	Hirata et al., 1996
Chlorella vulgaris LEB-104	45	Sydney et al., 2010
Chlorogleopsis sp	41	Ono and Cuello, 2007
Dunaliella tertiolecta SAG-13.86	36	Sydney et al., 2010
Euglena gracilis	65	Chae et al., 2006
Scenedesmus obliquus	49-51	Tang et al., 2011
Spirulina platensis LEB-52	50	Sydney et al., 2010

CO_2 can freely diffuse through the plasma membrane (Fig. 4.2.), suggesting that a CO_2 gradient is not built up along the plasmalemma (Sültemeyer and Rinast, 1996). Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) is the first enzyme in the Calvin cycle that assimilates CO_2 by converting it into 3-phosphoglycerate (3-PGA). Besides this carboxylase activity, rubisco displays oxygenase activity, using oxygen to form 3-PGA and phosphoglycolate. The latter is subsequently oxidatively metabolised via photorespiration, leading to a loss in CO_2 fixation (Graham and Wilcox, 2000). Since microalgae have a low CO_2-binding capacity of rubisco and the oxygenase activity of rubisco depends on the CO_2:O_2 ratio (Graham and Wilcox, 2000), an elevated intracellular CO_2 concentration is a prerequisite. Therefore, some microalgae possess carbon concentrating mechanisms (CCMs) that elevate the CO_2 level at the site of rubisco up to 1000-fold over that of the surrounding medium (Price et al., 2008). Microalgal CCMs involve (Fig. 4.2.): (1) cell membrane inorganic carbon transporters/pumps (e.g., HCO_3^-/Na^+ symports or ATP-driven uniports), (2) carbonic anhydrases (CA), (3) specialised cellular structures, and (4) calcification.

Figure 4.2. *Proposed models of inorganic carbon uptake and storage pathways in eukaryotic microalgae and prokaryotic cyanobacteria*
CO_2 can diffuse freely across membranes. Since bicarbonate and carbonate are charged ions, their uptake requires transporter molecules (T) and energy. Transformation of extracellular bicarbonate into CO_2 in the periplasmic space is therefore advantageous for the cells, as is cytoplasmic entrapment of CO_2. Similarly, carbonic anhydrase (CA) in chloroplasts (microalgae) and peroxisomes (cyanobacteria) may trap bicarbonate within the organelle and regenerate CO_2 adjacent to rubisco where it is needed for fixation. Carbonate can be stored at the cell wall while producing CO_2 via calcification.
Based on: Jansson and Northen, 2010; Mikhodyuk et al., 2008; Graham and Wilcox, 2000; Badger and Price, 1994.



An aspect of importance, for example for modelling, is the form of inorganic carbon which can be directly up-taken by algae (Fig. 4.2.). Carbon dioxide can cross cell membranes and thus can directly enter the cell by diffusion in both microalgae as cyanobacteria (Giordano et al., 2005a,b; Amoroso et al., 1998; Badger and Price, 1989). Bicarbonate can also be up-taken by certain microalgae and cyanobacteria, but this requires a transporter system or its prior conversion to CO_2 (Giordano et al., 2005a,b). Certain cyanobacteria can also utilise CO_3^{2-}, but only HCO_3^- accumulates in their cytoplasm (Mikhodyuk et al., 2008). To the best of our knowledge, no direct uptake of bicarbonate by eukaryotic microalgae have been reported. Bicarbonate and carbonate must be converted into CO_2 before these can be used by rubisco. Bicarbonate and CO_2 can be interconverted via the reversible reaction catalysed by CA (Lane et al., 2005):

$HCO_3^- + H^+ \quad <-> CO_2 + H_2O$ (E.4.9.)

Internal CA can be located in peroxisomes (only cyanobacteria) or in plastids and pyrenoids inside plastids (eukaryotics) (Fig. 4.2.). In some microalgae, external CA is always present whereas in others it is induced by low CO_2 levels or absent (Graham and Wilcox, 2000).

Another way microalgae can generate CO_2 from bicarbonate is to excrete H^+ across their cell membranes, which reacts with HCO_3^- to yield CO_2 and H_2O. Microalgae-induced calcification, i.e., the formation of recalcitrant calcium carbonate, can also generate CO_2 via reaction (E. 4.10.). At a higher pH, calcification can proceed without generating CO_2, according to reaction (E. 4.11.) (Jansson and Northen, 2010).

$Ca^{2+} + 2\ HCO_3^- \quad <-> \quad CaCO_3 + CO_2 + H_2O$ (E. 4.10.)

$Ca^{2+} + CO_3^{2-} \quad <-> \quad CaCO_3$ (E. 4.11.)

Calcification has been intensively studied, but the mechanistic details of this process are still poorly understood. It might confer a selective advantage in providing a protective shield against high light exposure, by offering a means of excreting toxic levels of intracellular calcium, enhancing nutrient uptake, serving as a buffer against pH rises in an alkaline environment, or by increasing the uptake of CO_2 (Jansson and Northen, 2010). The question arises of whether calcification occurs in flue gas-fed microalgae, since (1) at increased CO_2 levels, the CCM is not needed and cells might preferentially take up CO_2 rather than HCO_3^-, and (2) the pH will probably be lowered, impeding alkalinisation at the extracellular surface. However, cyanobacteria have

been found to calcify under elevated CO_2 levels (Obst et al., 2009). It still needs to be clarified to what extent processes such as Ca^{2+} efflux-generating extracellular alkalinisation, passive Ca^{2+}-binding and nucleation at the EPS layer (extracellular polymeric substances layer) or S layer, and CA activity at the EPS-layer are involved in this (Jansson and Northern, 2010).

4.3.1.3. CO_2 tolerance and effects on microalgae

Several microalgal species have shown good tolerance to sparging with gas containing 5 to 20 % CO_2, i.e., concentrations as in flue gas (Table 4.5.). Even tolerance up to 40 and 100 % of CO_2 has been reported (Matsumoto et al., 1997; Olaizola, 2003). The ability of certain microalgae to tolerate high CO_2 concentrations might be explained by a myriad of reasons. Firstly, when low gas flow rates are applied, even high CO_2 concentrations in the gas phase can still lead to low inorganic carbon loadings in the liquid phase and low concentrations of dissolved inorganic carbon in the reactor. Therefore, we should also consider the concentrations of dissolved CO_2 (and other flue gas compounds) that the microorganisms encounter, and not only in the concentration of a flue gas compound in the gas phase. Up to now, this information has been rarely reported in scientific literature. Secondly, CCMs may play a role. Inhibition of rubisco through acidification under high CO_2 conditions may be prevented by CA (Miyachi et al., 2003). Thirdly, with certain algal species, the addition of bases to compensate for CO_2 acidification enhances CO_2 tolerance. By this, microalgal growth can be sustained even at 100 % CO_2 (Olaizola, 2003), suggesting that it is mainly acidification that inhibits microalgal growth. By contrast, Soletto et al. (2008) attributed this inhibition to an increase in osmotic pressure. Xu (2004) reported that high CO_2 stress inhibited the efficiency of PSII. Overall, the CO_2 tolerance of microalgae is dependent on cell density (Chiu et al., 2008), pH (Olaizola, 2003), nutrients, light (Soletto et al., 2008) and species (Tang et al., 2011).

Carbon dioxide enrichment can affect the biochemical composition of algae in several ways. Reports on the effect of CO_2 on lipid content and composition are contradictory. Ota et al. (2009) found that at CO_2 concentrations between 20 to 50 %, the total fatty acid content of *Chlorococcum littorale* decreased. High CO_2 levels (30 to 50 %) have been shown to favour the accumulation of total lipids and polyunsaturated fatty acids in certain microalgae (Tang et al., 2011; Chiu et al., 2009).

This increase in polyunsaturated fatty acids has been explained by a relative decrease in oxygen concentration that might affect enzymatic desaturation (Vargas, 1998). Shifting *Chlorella* from limiting (air; 0.04 %) to higher (4 to 5 %) CO_2 concentrations increases the proportion of saturated fatty acids (Tsuzuki et al., 1990). Desaturation of fatty acids has been seen to occur more rapidly when the CCM is induced in *Chlamydomonas reinhardtii* (Pronina et al., 1998).

Xia and Gao (2005) observed that enriching the cultures of *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa* with CO_2 decreased nitrate reductase activity. They explained this by a modification of the nitrate reductase via protein phosphorylation because the phosphorylation level was higher in cells incubated in the presence of air enriched with CO_2 compared with air alone. Larsson et al. (1985) found that CO_2 addition led to an increased internal N content in *Scenedesmus obtusiusculus*. Flue gas enrichment has been observed to increase the chlorophyll *a*: chlorophyll *b* ratio in *Chlorella pyrenoidosa* but not in *Chlamydomonas reinhardtii* (Xia and Gao, 2005). An increased inorganic carbon:organic carbon ratio also increases the chlorophyll *a* content of microalgal bacterial flocs (Van Den Hende et al., 2011b). Elevating CO_2 concentrations can decrease CA activity (Xia and Gao, 2005). To date, little is understood in relation to the effect of CO_2 injection on the microalgal biomass composition.

Vice versa, microalgae may affect CO_2 solubility. Microalgal metabolites can modify the surface tension of the medium and thus, form an extra barrier to CO_2 transfer, decreasing CO_2 removal from flue gas (Grima et al, 1993). On the other hand, the production of external CAs may enhance the hydratation of CO_2 (Collett et al., 2011).

4.3.1.4. CO_2 removal by microalgae

Flue gas sparged into a microalgal reactor may have CO_2 removed by both fixation into microalgal biomass as well as dissolution into the water. Microalgal fixation of inorganic carbon from flue gas can be performed by: (1) conversion into biomass via photosynthesis followed by respiration (fixation as organic carbon) and (2) calcification (fixation as refractory carbon). A part of this removed carbon can leak as (1) extracellular organic compounds (up to 80 % of the carbon fixed by photosynthesis in natural systems and around 6-16 % in microalgal photobioreactors;

Hulatt and Thomas, 2010) including volatile organic compounds (VOCs), carbohydrates, polyamines, organohalogens and pheromones (Tarakhovskaya et al., 2007), or as (2) inorganic carbon via photorespiration (Richmond, 2004). A measured closed C-balance in a flue gas-sparged microalgae reactor by determining all organic and inorganic carbon in the gas, liquid and biomass, has not been published yet to the best of our knowledge. This indicates its difficulty. The main research on microalgal CO_2 fixation has focused on (1) species isolation, (2) optimisation of reactor design and operation, and (3) fundamental research on CCMs (Jansson and Northen, 2010).

Variation in the methodology for CO_2 removal rates, reactors and operating conditions (Table 4.5.) makes the comparison of the performance of microalgal strains difficult. Overall, the CO_2 removal rate depends on the gas aeration rate and CO_2 concentration (Ryu et al., 2009), light intensity (Li et al., 2011), light spectrum (Fan et al., 2007), photoperiod (Jacob-Lopes et al., 2008b), cell density (Jiang et al., 2011), temperature (Chinnasamy et al., 2009), reactor type (Kumar et al., 2011) and nutrients (Jin et al., 2006). In general, to obtain a high CO_2 removal rate via biomass fixation, the maximum microalgal growth rate should be aimed at by changing the above parameters to optimal conditions, i.e. exponential growth phase. In practice, the photon flux density and temperature will be the most difficult to optimise at a cost-effective way, especially during winters in temperature climates.

4.3.1.5. CO_2 credits and methods to assess CO_2 removal in algae reactors

In literature, a variety of methods assessing CO_2 removal in microalgal reactors have been published (Table 4.5.). Measuring the differences in CO_2 concentrations in the gas phases (e.g., Francisco et al., 2010) overestimates microalgal CO_2 uptake, since some of the carbon is dissolved in the culture medium. This has led to impressive carbon removal rates and up to 30 times higher CO_2 fixation:biomass productivity ratios (Fig. 4.3.). Additionally, measuring the dissolved carbon content of the influent and effluent gives a more realistic estimation (e.g., Van Den Hende et al., 2011a). This does not take into account the leaking of VOCs, but the latter is relatively low (in the order of 1 to 10 µg g^{-1} dry weight of algae; Walsh et al., 1998). Another method involves measuring biomass productivity together with the carbon content of the microalgae (e.g., Tang et al., 2011). This carbon content is often not measured but calculated from a CO_2 biofixation rate:biomass productivity conversion

factor of 1.88, which is derived from the molecular formula of microalgal biomass ($CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$) (Chisti, 2007). This formula assumes a 51.4 % carbon content of dry microalgae, which is a good average of the reported values ranging from 36 to 65 % (Table 4.4.). Calculating this conversion factor from several studies where the C content of microalgae was measured (Fig. 4.3.) shows that this factor can range from 1.81 to 2.37. The higher conversion factors might be due to calcification of microalgae.

According to several authors (e.g., Brennan and Owende, 2010), mitigation of CO_2 emissions from flue gas with microalgae may generate extra economic revenues by means of CO_2 credits. Here, we show that the method to assess CO_2 removal has large implications on the calculations of CO_2 credits. If only fixation into microalgal biomass is taken into account, the revenues of CO_2 credits will be small compared to the total production costs and definitely not a panacea for the high cost of algal production. For example, the current price for carbon credits of 15 € per ton CO_2 (Van Den Hende et al., 2011a) would result in 0.036 € per kg of dry biomass for microalgae containing 65 % carbon. This is only 0.9 % of the total microalgal production cost of around 4 € per kg of dry weight (Norsker et al., 2011). On the other hand, when the difference in CO_2 concentration in the in-gas and off-gas is used to assess CO_2 sequestration by a microalgal reactor, significant revenues via CO_2 credits can be achieved. For example, Jacob-Lopes et al. (2010) demonstrated that the total CO_2 sequestration from the gas phase in a microalgae reactor was 32 times that of the CO_2 fixation via biomass. This means that 1.14 € of carbon credits per kg of microalgal biomass (32 times 0.036 € per kg) could be obtained or 29 % of the total microalgae production cost of around 4 € per kg of dry weight. This large difference is mainly due to the dissolution of CO_2 in the algal medium combined with a low hydraulic retention time and discharge of an effluent rich in inorganic carbon. Currently, there are no limits for the inorganic carbon content of discharged effluent (but the limits for pH and conductivity may influence it indirectly). The question remains on whether discharging dissolved inorganic carbon into surface waters would be accepted by policy makers as a carbon fixation technology. In conclusion, there is a huge need for a clear and global method to assess the carbon credits (and similarly NO_x and SO_x credits) that can be obtained by flue gas injection into a microalgae reactor.

***Table 4.5**. Removal efficiency (RE) and removal rate (RR) of CO_2, SO_2 and NO, and biomass productivity of several microalgae strains (Table continues on next pages)*

Species	PPFD (μmol m^{-2} s^{-1})	T (°C)	Reactor type[a]	V reactor (L)	Gas flow rate (L h^{-1})	Gas loading (vvm)	CO_2 conc. (%v)	CO_2 RE (%)	CO_2-C RR (g C L^{-1} day^{-1})	Method[b]
Aphanothece	150	30	BC	2.0	120	1.00	15	nd[c]	0.14	A
microscopic	150	30	BC	2.0	120	1.00	15	nd	4.58	C
	150	35	AL	2.4	180	1.00	15	nd	0.39	C
	150	35	BC	2.4	180	1.00	15	nd	0.15	C
	150	30	T	3.0	180	1.00	15	nd	11.00[m]	A
Chlorella	180	25	E	0.80	12	0.25	0.03	nd	0.037[m]	/
pyrenoidosa	180	25	E	0.80	12	0.25	5	nd	0.067[m]	E
SJTU-2	180	25	E	0.80	12	0.25	10	nd	0.071[m]	E
	180	25	E	0.80	12	0.25	20	nd	0.061[m]	E
	180	25	E	0.80	12	0.25	30	nd	0.041[m]	E
	180	25	E	0.80	12	0.25	50	nd	0.029[m]	E
Chlorella sp.	100	nd	T	0.60	3.6	0.1	5	31	0.192	C
	100	nd	T	0.60	7.2	0.2	2	nd	0.141	C
	100	nd	T	0.60	7.2	0.2	5	20	0.159	C
Chlorella sp.	300	26	E	0.8	12	0.25	15	16	4.69	A
(marine)	300	26	E	0.8	12	0.25	10	20	3.81	A
	300	26	E	0.8	12	0.25	5	27	2.59	A
	300	26	E	0.8	12	0.25	2	58	2.14	A
Chlorella sp.	450	25	GWB	0.05	1.5	0.5	15	nd	nd	/
KR-1	450	25	GWB	0.05	1.5	0.5	15	nd	nd	/
	450	25	GWB	0.05	1.5	0.5	15	nd	nd	/
	450	25	GWB	0.05	1.5	0.5	15	nd	nd	/
C. sp. P12[d]	Out[e]	Out	OTL	330	9-35 m^3	0.5-1.8	6-8	39	1.79	A
Chlorella sp.	110	35	SP	70	nd	nd	13	nd	nd	/
T-1	110	35	SP	0.6	30	0.83	15	nd	nd	/
	110	35	SP	0.6	30	0.83	15	nd	nd	/
	110	35	SP	0.6	30	0.83	15	nd	nd	/
Chlorella	200	nd	CHF	9	180	0.33	1	70	1.70	A
vulgaris	130	25	M	5.6	75	0.22	1	nd	0.075	A
	130	25	BC	5.6	75	0.22	1	nd	0.038	A
	130	25	AL	5.6	75	0.22	1	nd	0.038	A
	130	25	MC	5.6	75	0.22	1	nd	0.012	A
	150	30	T	3.0	180	1.00	15	nd	7.00[m]	A
	200	27	BC	1	nd	nd	15	nd	0.130	C
	38[f]	30	T	1.80	32.4	0.30	12	nd	nd	/
	42[f]	30	Bi-Fl	8.0	nd	nd	5	nd	0.068	B

NO conc. (mg Nm^{-3})	NO RE (%)	NO RR (g NO L^{-1} day^{-1})	SO_2 conc. (mg Nm^{-3})	SO_2 RE (%)	SO_2 RR (g SO_2 L^{-1} day^{-1})	Biomass productivity (mg L^{-1} day^{-1})	Reference
0	/	/	0	/	/	nd[c]	Jacob-Lopes et al., 2010
0	/	/	0	/	/	nd	Jacob-Lopes et al., 2010
0	/	/	0	/	/	0.77	Jacob-Lopes et al., 2009
0	/	/	0	/	/	0.301	Jacob-Lopes et al., 2009
0	/	/	0	/	/	0.754	Francisco et al., 2010
0	/	/	0	/	/	0.065	Tang et al., 2011
0	/	/	0	/	/	0.133	Tang et al., 2011
0	/	/	0	/	/	0.144	Tang et al., 2011
0	/	/	0	/	/	0.121	Tang et al., 2011
0	/	/	0	/	/	0.075	Tang et al., 2011
0	/	/	0	/	/	0.054	Tang et al., 2011
0	/	/	0	/	/	0.336	Ryu et al., 2009
0	/	/	0	/	/	0.295	Ryu et al., 2009
0	/	/	0	/	/	0.335	Ryu et al., 2009
0	/	/	0	/	/	0.369	Chiu et al., 2008
0	/	/	0	/	/	0.458	Chiu et al., 2008
0	/	/	0	/	/	0.491	Chiu et al., 2008
0	/	/	0	/	/	0.528	Chiu et al., 2008
0	/	/	286	/	/	0.78	Lee et al., 2002
0	/	/	172	/	/	1.24	Lee et al., 2002
30	/	/	229	/	/	0.71	Lee et al., 2002
402	/	/	0	/	/	nd/ tol/	Lee et al., 2002
27-60[x]	nd	nd	nd	nd	nd	3.8	Doucha et al., 2005
201[x]	nd	nd	29	nd	nd	nd / tol.	Maeda et al., 1995
80[x]	nd	nd	57	nd	nd	nd / tol.	Maeda et al., 1995
20[x]	nd	nd	14	nd	nd	nd / tol.	Maeda et al., 1995
40[x]	nd	nd	29	nd	nd	nd / tol.	Maeda et al., 1995
0	/	/	0	/	/	nd	Cheng et al., 2006
0	/	/	0	/	/	nd	Fan et al., 2007
0	/	/	0	/	/	nd	Fan et al., 2007
0	/	/	0	/	/	nd	Fan et al., 2007
0	/	/	0	/	/	nd	Fan et al., 2007
0	/	/	0	/	/	0.482	Francisco et al., 2010
0	/	/	0	/	/	1.85	Jin et al., 2006
134	nd	nd	172	nd	nd	nd	Radmann and Costa, 2008
0	/	/	0	/	/	0.81	Sydney et al., 2010

Species	PPFD (μmol m^{-2} s^{-1})	T (°C)	Reactor type[a]	V reactor (L)	Gas flow rate (L h^{-1})	Gas loading (vvm)	CO_2 conc. (%v)	CO_2 RE (%)	CO_2-C RR (g C L^{-1} day^{-1})	Method[b]
C. littorale[g]	250	25	Bat	20	60	0.05	20	nd	0.246	A
C. melorae[h]	nd	50	Bat	nd	nd	nd	2-6.5	nd	nd	/
C. cald.[i]	nd	50	Bat	nd	nd	nd	2-6.5	nd	nd	/
Dunaliella	150	30	T	3.0	180	1.00	15	nd	5.82 [m]	A
tertiolecta	149[f]	25	PFA	7.85	9.0	0.019	15	nd	nd	/
	149[f]	25	CFA	7.85	9.0	0.019	15	nd	nd	/
	149[f]	25	BCS	7.85	9.0	0.019	15	nd	nd	/
	149[f]	25	BCG	7.85	9.0	0.019	15	nd	nd	/
	149[f]	25	2S-BC	15.7	9.0	0.019	15	nd	nd	/
	42[f]	25	Bi-Fl	8.0	nd	nd	5	nd	0.074	B
E. gracilis[j]	490	28	L	1000	1800	0.03	11	nd	nd	E
G. partita[k]	nd	50	Bat	nd	nd	nd	2-6.5	nd	nd	/
MaB- flocs	100	20	T	4	0.60	0.0025	12	49	0.099	D
M. aerusino.[l]	200	27	BC	1	nd	nd	15	nd	0.134	C
M. ichthyob.[p]	200	27	BC	1	nd	nd	15	nd	0.142	C
P. tricornit.[q]	150	30	T	3.0	180	1.00	15	nd	0.59[m]	A
Phorm. sp.[r]	150	30	T	3.0	180	1.00	15	nd	7.39[m]	A
Scenedesmus	150	30	T	3.0	180	1.00	15	nd	4.60[m]	C
obliquus	120	28	PBR	0.5	6	0.2	20	40	nd	A
	38[f]	30	T	1.80	32.4	0.30	12	nd	nd	/
Scenedesmus	180	25	E	0.80	12	0.25	0.03	nd	0.041[m]	E
obliquus	180	25	E	0.80	12	0.25	5	nd	0.078[m]	E
SJTU-3	180	25	E	0.80	12	0.25	10	nd	0.079[m]	E
	180	25	E	0.80	12	0.25	20	nd	0.067[m]	E
	180	25	E	0.80	12	0.25	30	nd	0.041[m]	E
	180	25	E	0.80	12	0.25	50	nd	0.029[m]	E
Scenedesmus	120	28	AL	0.5	6	0.2	20	63	nd	A
obliquus	150	26	AL	100	1200	0.5	18	20	nd	A
WUST4	150	26	AL	100	1200	0.1	12	67	nd	A
	150	26	AL	100	1200	0.1	18	40	nd	A
Scenedesmus	200	27	BC	1	nd	nd	15	nd	0.167	C
sp.	200	nd	AL	1.0	18	0.30	15	nd	nd	/
	160	nd	Bat	94	120	0.021	20	nd	nd	/
Spirulina sp.	42[f]	30	Bi-Fl	8.0	nd	nd	5	nd	0.087	B
	38[f]	30	T	1.80	32.4	0.30	12	nd	nd	/
S. nidulans[s]	38[f]	30	T	1.80	32.4	0.30	12	nd	nd	/

NO conc. (mg Nm^{-3})	NO RE (%)	NO RR (g NO L^{-1} day^{-1})	SO_2 conc. (mg Nm^{-3})	SO_2 RE (%)	SO_2 RR (g SO_2 L^{-1} day^{-1})	Biomass productivity (mg L^{-1} day^{-1})	Reference
0	nd	nd	0	nd	nd	0.53	Kurano et al., 1995
27-80	nd	nd	821-918	nd	nd	nd/ tol.	Kurano et al., 1995
27-80	nd	nd	821-918	nd	nd	nd/ tol.	Kurano et al., 1995
0	/	/	0	/	/	0.367	Francisco et al., 2010
134	51-75[n]	nd	0	/	/	nd	Nagase et al., 1998
134	57-96[n]	nd	0	/	/	nd	Nagase et al., 1998
134	20-26[n]	nd	0	/	/	nd	Nagase et al., 1998
134	35-65[n]	nd	0	/	/	nd	Nagase et al., 1998
134	90[n]	nd	0	/	/	nd	Nagase et al., 1998
0	/	/	0	/	/	0.65	Sydney et al., 2010
nd	nd	nd	nd	nd	nd	0.114	Chae et al., 2006
27-80	nd	nd	821-918	nd	nd	nd/ tol.	Kurano et al., 1995
400	87	0.487	572	99	0.74	0.181	VD Hende et al., 2011a
0	/	/	0	/	/	1.90	Jin et al., 2006
0	/	/	0	/	/	2.14	Jin et al., 2006
0	/	/	0	/	/	0.007	Francisco et al., 2010
0	/	/	0	/	/	0.415	Francisco et al., 2010
0	/	/	0	/	/	0.655	Francisco et al., 2010
0	/	/	0	/	/	nd	Li et al., 2011
134	nd	nd	172	nd	nd	nd	Radmann and Costa, 2008
0	/	/	0	/	/	0.083	Tang et al., 2011
0	/	/	0	/	/	0.158	Tang et al., 2011
0	/	/	0	/	/	0.155	Tang et al., 2011
0	/	/	0	/	/	0.134	Tang et al., 2011
0	/	/	0	/	/	0.081	Tang et al., 2011
0	/	/	0	/	/	0.056	Tang et al., 2011
0	/	/	/	/	/	nd	Li et al., 2011
201[x]	nd	nd	572	nd	ndn	nd	Li et al., 2011
201[x]	nd	nd	572	nd	nd	nd	Li et al., 2011
201[x]	nd	nd	572	nd	nd	nd	Li et al., 2011
0	/	/	0	/	/	2.01	Jin et al., 2006
201	80-85	0.023	0	nd	nd	0.35[m]	Santiago et al., 2010
429-536	nd	nd	2073	nd	nd	nd	Westerhof, 2010
0	/	/	0	/	/	0.44	Sydney et al., 2010
134	nd	nd	172	nd	nd	nd	Radmann and Costa, 2008
134	nd	nd	172	nd	nd	nd	Radmann and Costa, 2008

Notes to Table 4.5.

[a] Reactor type: PBR: photobioreactor; L: L-shape PBR; CHF: cylindrical hollow fibre PBR; E: Erlenmeyer; OTL: open thin layer reactor; M: membrane PBR; BC: bubble column PBR; AL: airlift PBR; MC: membrane contactor; T: tubular PBR; GWB: gas washing bottle; SP: spinner flask; PFA: parallel flow airlift PBR; CFA: counter flow airlift PBR; BCS: bubble column stainless steel sparger PBR; BCG: bubble column glass ball sparger PBR; 2S-BC: two serial BCG; Bi-Fl: Bio-Floc Fermentor; [b] Method used to assess CO_2 removal: A: diff gas CO_2: values based on difference in CO_2 concentration of ingas and off gas; B: diff gas CO_2 cr: values based on difference in CO_2 concentrations of ingas and off gas corrected for CO_2 removal in sterilized medium without algae; C: P x %C theoretical: biomass productivity combined with the theoretical C content of biomass; D: diff gas CO_2 + diff. TIC liq.: value based on difference in CO_2 concentration of ingas and off gas combined with difference in total inorganic carbon content of influent and effluent; E: P x % C measured: biomass productivity combined with the measured C content of biomass; [c] nd: no data; [d] *Chlorella vulgaris* sp. P12; [e] out: outdoor; [f] Light intensity was calculated from W m^{-2} or lux using conversion of Mc Cree (1981); [g] *Chlorococcum littorale*; [h] *Cyanidioschyzon melorae*; [i] *Cyanidium caldarium;* [j] *Euglena gracilis*; [k] *Galderia partitia*; [l] *Microcystis aerusinosa*; [m] Only maximum values were reported; [n] Values based on difference in NO in ingas and NO in off gas; [0] Values based on difference in NO in ingas and both NO and NO_2 in off gas; [p] *Microcystis ichtyoblabe*; [q] *Phaedactylum tricornitum*; [r] *Phormidium sp.*; [s] *Synechococcus nidulans*; [t] tol.: tolerance; [x]: NO_x.

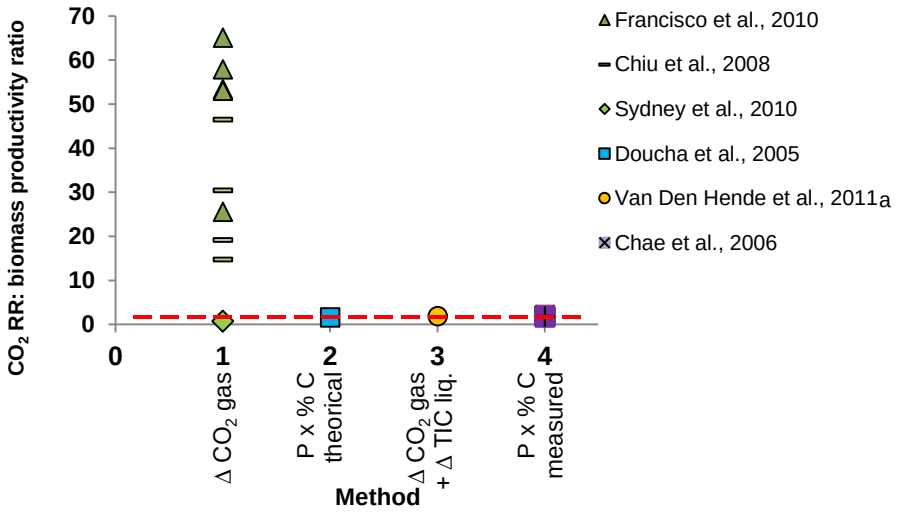


Figure 4.3. *The CO_2 removal rate (RR): biomass productivity ratio calculated from several studies, where the CO_2 RR was assessed by different experimental methods*
1: the difference in CO_2 concentrations of in-gas and off-gas; 2: biomass productivity combined with the theoretical carbon content; 3: the difference in CO_2 concentrations of in-gas and off-gas combined with the difference in total inorganic carbon content from influent and effluent; 4: biomass productivity combined with the analysed carbon content.

4.3.2. Nitrogen oxides

4.3.2.1. NO_x solubility in aqueous solutions

In general, NO_x species emitted from incineration processes consist of 95 % NO and 5 % NO_2 (Wang et al., 2008b). The colourless gas NO, a relatively stable free radical, is poorly soluble in water (0.032 g L^{-1} at 1 atm and 25 ºC; Dora et al., 2009), leading to low absorption rates in aqueous media. NO_2, a red-brown gas, is formed from the oxidation of NO as follows:

$$2\ NO_{(g)} + O_2 \quad <-> \quad 2\ NO_{2\,(g)} \qquad \text{(E. 4.12.)}$$

NO_2 has a 6000-times higher solubility than NO (213.0 g L^{-1} at 25 °C and 1 atm; Dora et al., 2009). Other NO_x are the black solid N_2O_3, the red-brown gas N_2O_4, the colourless gases N_2O_2 and NO_2, and the white solid N_2O_5, but they exist only at very small concentrations in flue gas (EPA, 1999; Van Loo and Koppejan, 2008). When any of these NO_x dissolve in water, they form nitric acid (HNO_3) or nitrous acid (HNO_2). For NO and NO_2, this is a result of (Stumm and Morgan, 1981; Williams, 2011):

$$NO_{(g)} \quad <-> \quad NO_{(aq)} \qquad \text{(E. 4.13.)}$$

$$NO_{(aq)} + H_2O \quad <-> \quad HNO_2 \qquad \text{(E. 4.14.)}$$

$$NO_{2(g)} \quad <-> \quad NO_{2(aq)} \qquad \text{(E. 4.15.)}$$

$$2\ NO_{2(aq)} + H_2O \quad <-> \quad HNO_2 + HNO_3 \qquad \text{(E. 4.16.)}$$

$$3\ HNO_2 \quad <-> \quad HNO_3 + 2\ NO_{(aq)} + H_2O \qquad \text{(E. 4.17.)}$$

In microalgal reactors, the pH is normally higher than 4, and HNO_3 and HNO_2 are mainly present as NO_3^- and NO_2^-, respectively (Stumm and Morgan, 1981; pK at 25°C and 1 atm):

$$HNO_2 \quad <-> \quad NO_2^- + H^+ \quad (pK= 3.29) \qquad \text{(E. 4.18.)}$$

$$HNO_3 \quad <-> \quad NO_3^- + H^+ \quad (pK= -1.3) \qquad \text{(E. 4.19.)}$$

Recently, it has been found that ionisation of N_2O_4 is a key step in the hydrolysis of NO_2 in a water film (Miller et al., 2009):

$$2\ NO_{2\,(g)} \quad <-> \quad N_2O_{4\,(g)} \qquad \text{(E. 4.20.)}$$

$$N_2O_4 + H_2O \quad <-> \quad NO^+NO_3^- + H_2O \quad <-> \quad HNO_2 + HNO_3 \qquad \text{(E. 4.21.)}$$

In most algal reactors, the medium contains oxygen, and NO and NO_2 may react as follows:

$$4\ NO_{(aq)} + 2\ H_2O + 3\ O_2 \quad <-> \quad 4\ HNO_3 \qquad \text{(E. 4.22.)}$$

$$4\ NO_{2(aq)} + 2\ H_2O + O_2 \quad <-> \quad 4\ HNO_3 \qquad \text{(E. 4.23.)}$$

When solar or artificial light is provided, NO_x chemistry is complicated by the photolysis of nitrogenous compounds and the reaction with photochemical oxidants (Skalska, 2010):

$NO_{(g)} + O_3$ <-> $NO_{2(g)} + O_2$ (E. 4.24.)

NO_2 + photon(λ< 400 nm)<-> NO + O (E. 4.25.)

$NO_2 + {}^{\cdot}OH$ <-> HNO_3 (E. 4.26.)

$NO_{(aq)} + 0.5\ O_2$ <-> $NO_{2(g)}$ (E. 4.27.)

Moreover, when flue gas containing both NO_2 and SO_2 is sparged in microalgae reactors, the latter two may react with each other as follows:

$NO_2 + SO_2$ <-> $NO + SO_3$ (E. 4.28.)

Clearly, the chemistry of flue gas NO_x in microalgae reactors and their interactions with other chemical species and light is very complex. Overall, the solvability of NO in water is the rate limiting step, but can be increased by chelators (see Section 4.3.2.4 Removal of NO_x by microalgae).

4.3.2.2. NO_x uptake and metabolisation pathways by microalgae

After carbon, nitrogen is the most important nutrient for algal production. Whereas the Redfield C:N ratio of microalgal biomass is 6.66 (molar), this ratio varies in practice among species, with reported values ranging between 3 and 17 (Geider and La Roche, 2002). In general, microalgae can take up nitrogen in several forms: NH_4^+, NO_3^-, NO_2^-, NO and N_2 (Fig. 4.4.). Both nitrate and nitrite reduction is tightly coupled to energy supply from the photosynthetic electron transfer or external organic carbon (Graham and Wilcox, 2000).

Latest research on NO_x uptake pathways by microalgae has been limited to a few publications, mainly focusing on NO uptake by *Dunaliella tertiolecta*. In 1997, Nagase et al. found that the presence of both algal cells and oxygen enhanced the removal of NO in microalgae reactors. Without light, NO removal did not occur in the algal cultures in the absence of O_2. The latter authors suggested that with light NO first dissolved in water, after which it was oxidised and assimilated by the cells:

$NO_{(g)}$ <-> $NO_{(aq)}$ (E. 4.29.)

$NO_{(aq)}$ + 0.5 O_2 <-> NO_2 (E. 4.30.)

Brown (1996) found that adding 201 mg NO Nm^{-3} to a *Monoraphidium minutum* culture led to double the concentration of nitrite and delayed nitrate

utilisation early in the growth curve. This implies that some of the NO dissolved and was available as an N-source for the microalgae. Nagase et al. (2001) concluded that little NO was oxidised in the medium before its uptake by algal cells. Since NO is a small nonpolar molecule, they suggested that NO can diffuse through cell membranes. Moreover, they concluded that NO was preferentially used as a source for microalgal growth rather than nitrate. Nagase et al. (2001) performed their experiments with monocultures of *Dunaliella tertiolecta*, but it was not mentioned if these tests were performed in axenic conditions. Therefore, these experiments should be repeated in axenic conditions to confirm if bacteria played a role in this NO removal in algal cultures. These researchers based these conclusions on the nitrogen balance calculations. The exact mechanisms through which microalgae use NO_x are still to be proven with more accurate techniques such as nitrogen isotopes.

Microalgae can also produce and emit NO. In several microalgal species, this NO is produced if nitrate is present and ammonium absent, and is mediated by nitrate reductase (Mallick et al., 1999). In *Scenedesmus obliquus*, nitrite accumulation is responsible for this NO production (Sakihama et al., 2001). In general, microbial NO synthesis can be achieved from nitrite, nitrate or L-arginine (Vermeiren et al., 2009). The mechanisms by which microalgae produce NO require further investigation.

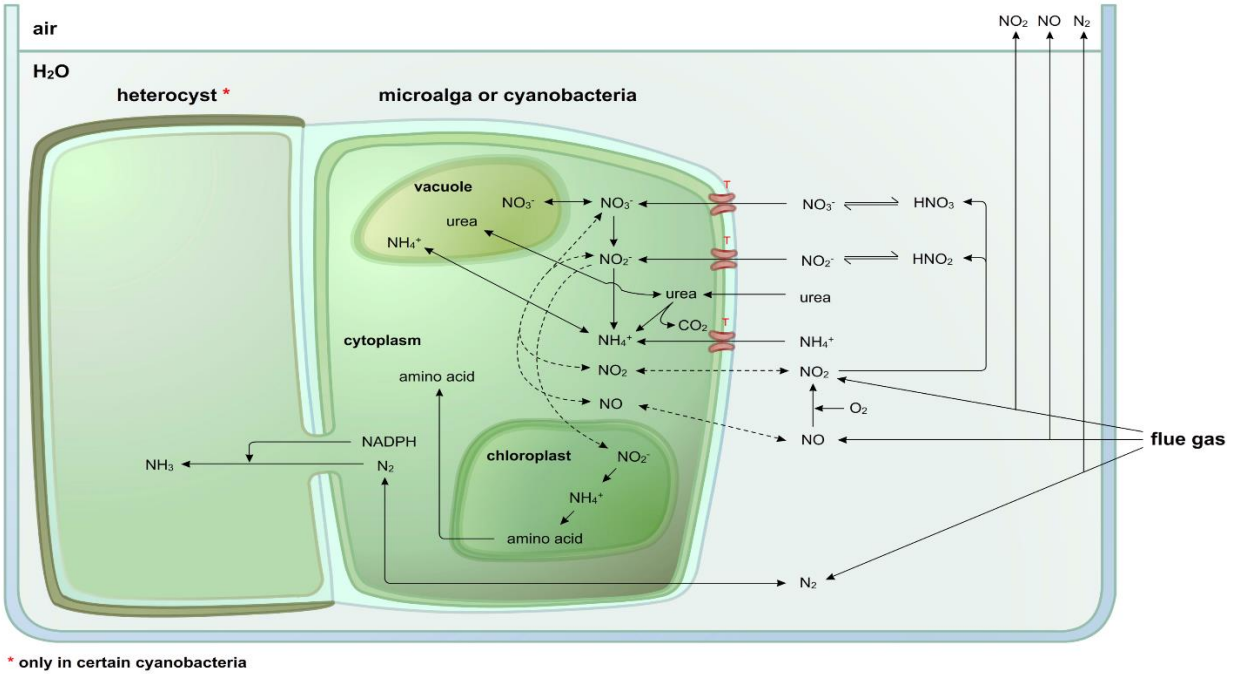
4.3.2.3. NO_x tolerance and effects on microalgae

The tolerance of microalgae to NO_x depends on microalgal cell density (Yoshihara, 1996), NO_x concentration (Doucha et al., 2005), NO_x gas flow rate (Li et al., 2011), reactor type (Nagase et al., 1998) and species (Radmann and Costa, 2008) (Table 4.5.).

Until 1987, nitric oxide was widely considered to be just a toxic gas. By the late 1990s, NO was identified as an important messenger in plant defence signalling against microbial pathogens and in growth (e.g., inhibiting photosynthetic ATP synthesis), development, photo-inhibition and death of plants (Delledonne et al., 1998). Whether NO also plays a similar role in microalgae and whether addition of NO at concentrations present in flue gas can affect this are unknown. Nagase et al. (2001) found that the content of lipids, starch and protein of microalgae were similar with and without the addition of NO. To date, the effect of NO on the composition of microalgae has not been elaborated in detail.

Figure 4.4. *Proposed models for nitrogen uptake and storage by eukaryotic microalgae and prokaryotic cyanobacteria*

Following uptake by a membrane transport system (T), ammonium can be directly converted into reduced organic nitrogen and utilised in protein production. By contrast, nitrate needs to be reduced to nitrite and ammonium by nitrate reductase. NO is suggested to freely diffuse through the cell membrane and can be assimilated. NO can also be produced by microalgae from nitrite. Certain cyanobacteria can fix N_2 in a heterocyst adjacent to a vegetative cell. Diffusion of N_2 only occurs via vegetative cells, since heterocysts have thick cell walls that prevent O_2 diffusion. Dotted lines indicate pathways which need further investigation. Based on: Nagase, 2001; Graham and Wilcox, 2000; Stumm et al., 1981.



4.3.2.4. Removal of NO_x by microalgae

The rate limiting step for NO removal in bioreactor systems is the dissolution of NO into the microalgal culture medium (Jin et al., 2005). The addition of a chelator greatly improves NO removal in a *Scenedesmus* culture (Jin et al., 2008). Well known chelates able to form stable metal complexes are ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid (NTA), methyliminodiacetic acid (MIDA) and dimercaptopropanesulfonic acid (DMPS). Fe(II)EDTA particularly reacts rapidly with the absorbed NO gas to form stable metal-nitrosyl complexes:

$NO_{(g)} + Fe(II)EDTA \quad <-> Fe(II)EDTA\text{-}NO_{(aq)}$ (E. 4.31.)

Flue gases and microalgal cultures contain dissolved oxygen. In the presence of dissolved oxygen, Fe(II)EDTA is easily and irreversiably oxidised to Fe(III)EDTA in bacterial systems (Jin et al., 2008):

$4\ Fe(II)EDTA^{2-} + O_2 + 4H^+ \quad <-> Fe(III)EDTA^- + 2\ H_2O$ (E. 4.32.)

Since Fe(III)EDTA can no longer chelate NO (Jin et al., 2008), it decreases the process efficiency. Interestingly, in a microalgal NO removal system, constant NO removal was maintained over a long duration and a certain fraction of Fe(II)EDTA remained without being oxidised to Fe(III)EDTA because of the existence of a reversible oxidation-reduction balance between Fe(II)EDTA and Fe(III)EDTA (Santiago et al., 2010). So, Fe(II)EDTA can be regenerated in microalgal cultures, but this microalgal Fe(II) regeneration is species dependent. Since a long exposure to sunlight can destabilise the iron EDTA (Lockhart and Blakeley, 1975), Fe(II)EDTA addition to microalgae reactors may be too costly. Moreover, EDTA is a micropollutant. Therefore, the *in situ* production of iron siderophores, acting as a biochelator, seems advantageous and merits further investigation.

Apart from the CO_2 credits, economical revenue can also be generated from NO_x credits in certain countries (e.g., the USA and the Netherlands). The NO_x trade tariffs are higher than those for CO_2 (e.g., in the Netherlands, 100 *vs* 15 € ton^{-1} resp.) (Smit, 2011). Nevertheless, NO_x removal in microalgae reactors does not lead to important revenues, since removal rates are low. For example, Van Den Hende et al. (2011a) reported a removal of 0.74 g NO_x $m^{-3}_{reactor}$ day^{-1}, leading to a maximum of 0.027 € $m^{-3}_{reactor}$ $year^{-1}$ or 0.00041 € kg^{-1} microalgal bacterial biomass (VSS) (applying the Dutch NO_x tariff of 0.1 € per kg NO_x; Smit, 2011).

4.3.3. Sulphur oxides

4.3.3.1. SO_2 solubility in aqueous solutions

SO_2 is a colourless gas with a high solubility in water (22.971 g per 100 g H_2O at 0 °C, to 5.881 per 100 g H_2O gat 40 °C; Weil and Sandler, 1997). After SO_2 dissolves in water, it forms a slightly acidic aqueous solution of H_2SO_3 (Stumm and Morgan, 1981):

$SO_2 + H_2O$ <-> H_2SO_3 (E. 4.33.)

This acidity is assumed to be due to the formation and subsequent ionisation of H_2SO_3. The main form is SO_3^{2-} (sulphite) at pH 6 or above; HSO_3^- (bisulphite) is prominent at pH 2 to 6 (25 °C and 1 atm; Stumm and Morgan, 1981).

SO_3 can also be present in the flue gas (2 to 4 % of the sulphur in flue gas; Niessen, 2002). SO_3 quickly reacts with water to form sulphuric acid (H_2SO_4). The latter can also be formed from the oxidation of H_2SO_3:

$SO_2 + ½\ O_2$ <-> SO_3 (E. 4.35.)

$SO_3 + H_2O$ <-> H_2SO_4 (E. 4.36.)

$H_2SO_3 + ½\ O_2$ <-> H_2SO_4 (E. 4.37.)

At a pH above 1.9, SO_4^{2-} (sulphate) is the major species (25 °C and 1 atm; Stumm et al., 1981).

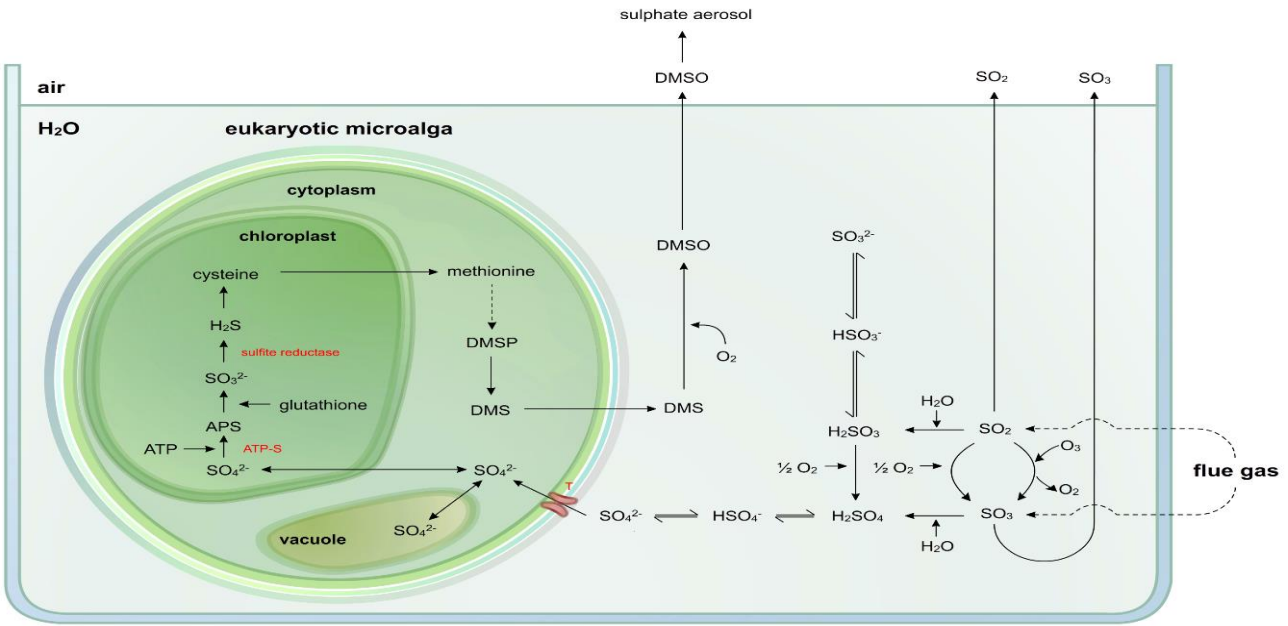
3.3.2. SO_2 uptake and metabolisation pathways by microalgae

Sulphur, an essential component of the amino acids cysteine and methionine, and S-containing thylakoid lipids, is undispensable for microalgal growth (Graham and Wilcox, 2000). Freshwater algae contain around 0.15 to 1.96 %w of sulphur by dry weight (Blaker et al., 1989). As far as it is known at this stage, microalgae acquire sulphur by taking up sulphate into the cytoplasm, for example, by means of high-affinity sulphate transporter systems (Giordano et al., 2005a,b) (Fig. 4.5.). Sulphate is transported into the plastids or, if present in excess, stored in vacuoles. This stabile sulphate ion is reduced via activation by ATP to 5′-adenylsulphate (APS), catalysed by ATP sulphurylase (ATP-S). APS is then reduced by APS reductase to sulphite (SO_3^{2-}). The produced sulphite is further reduced by sulphite reductase to sulphide (S^{2-}), which is immediately incorporated into cysteine.

Figure 4.5. *Proposed models for sulphur uptake and storage by both prokaryotic cyanobacteria and eukaryotic microalgae*

Flue gas compounds SO_2 and SO_3 are converted into sulphate, which is transported into the cell and if present in excess, stored in vacuoles. In the chloroplast, sulphate is reduced to APS, sulphite and sulphide, which is immediately incorporated into cysteine and methionine. Microalgae can excrete DMS, which is oxidised to DMSO and produces sulphate aerosols in the atmosphere.

T: transporter; APS: 5'-adenylsulphate; ATP-S: ATP sulphurase; DMSP: dimethylsulphide proprionate; DMS: dimethyl sulphide; DMOS: dimethyl sulphoxide. Based on: Giordano et al., 2005a,b; Graham and Wilcox, 2000; Genium, 1999; IARC, 1992; Stumm et al., 1981.



On the other hand, S-compounds can also be released by microalgae (Giordano et al., 2005b; Fig. 4). For example, *Emiliana sp.* and *Phaecystis* sp. generate dimethyl sulfio proprionate (DMSP) for use in osmoregulation or as a cryoprotectant in cold regions (Graham and Wilcox, 2000). This can then be converted into volatile dimethyl sulphide (DMS), which is released from the cells and oxidised to dimethyl sulphoxide (DMSO) to form sulphate aerosols. These aerosols promote an increase in cloud cover and an increase in the earth's albedo (fraction of incident radiation reflected back into space). DMS can be 29 % of the total sulphate assimilation of microalgae and can also generate SO_2 via reactions with NO_3 and OH (Giordano et al., 2005b).

4.3.3.3. SO_2 tolerance and effects on microalgae

Sulphur dioxide has been shown to be remarkably toxic to some microalgal species (Lee et al, 2000), but well tolerated by others (Table 4.5.). Both pH (Yang et al., 2004) and bisulphite concentrations (Bake et al., 1983) play a role in the tolerance of microalgae to SO_2. Biochemical effects of SO_2 arise from its unique ability to act as a reducing or oxidising agent. In plants, SO_2 is known to interfere directly with photosynthetic CO_2 fixation (competitive inhibition of rubisco by SO_3^-) and energy metabolism (inhibition of mitochondrial ATP production by SO_3^-), and indirectly via the formation of sulphites and organic sulphonates (Malhotra and Hocking, 1975). A few mechanisms leading to toxicity have been revealed specifically for microalgae. In an *Ankistrodesmus* sp. culture, bisulphite (100 mg L^{-1}) blocked the membrane transport of α-amino-^{14}C-isobutyric acid at pH 5 but not at pH 7 (Bake et al., 1983). Thus, toxicity to bisulphite is enhanced in acidic conditions. Yang et al. (2004) found that at low concentrations (< 104 mg sodium bisulphite L^{-1}) bisulphite was utilised as an S-source by *B. braunii* after oxidation of bisulphite to sulphate, but high concentrations of bisulphite (> 104 mg sodium bisulphite L^{-1}) were toxic. It was suggested that in the conversion of bisulphite into sulphate, superoxide anions, hydroxyl radicals and hydrogen peroxide were formed. These highly oxidative molecular species damaged membranes and pigment, causing peroxidation of membrane lipids and bleaching of chlorophyll, thus inhibiting the growth of *B. braunii*. This is consistent with the mechanism of bisulphite toxicity in lichens and higher plants (Giordano et al., 2005b).

Several strategies have been tested to lower the toxicity of SO_2 sparging on microalgae. Keeping the pH above 6 to prevent formation of sulphites by, for example, the addition of alkaline solutions has been successful (Lee et al., 2000a). Using acidophilic species such as *Galdiera partita* (Kurano et al., 1995) is another strategy.

To the best of our knowledge, research performed on the effect of SO_2 on the biochemical composition of microalgae is limited to the finding that the structure of hydrocarbons produced by *B. braunii* growing on different S-sources (including bisulphite and sulphite) showed no large differences (Yang et al., 2004).

4.3.3.4. SO_2 removal by microalgae

Due to the higher solubility of SO_2 in aqueous media and due to the higher SO_2 concentrations in flue gas, the revenues from SO_2 trading are expected to be higher than those from NO_x. In the USA, an SO_2 emission trade system was introduced in 1994, with prices ranging between 48 and 152 € per kg of SO_2 (Kessels and Hennesy, 2004). Data on removal rates of SO_2 in flue gas sparged reactors is very limited (Table 4.5.).

In a lab-scale microalgal bacterial flocs reactor fed with sewage and sparged with flue gas containing 572 mg Nm^{-3} SO_2 at a low gas flow rate of 0.0050 vvm, we observed an SO_2 removal rate of 3.2 ± 0 mg SO_2 L^{-1} day^{-1} and a slight increase in the sulphate concentration of wastewater from 50.7 ± 5.5 to 54.9 ± 2.6 mg L^{-1} (unpublished data from experiments of Van Den Hende et al., 2011a). Due to the low gas flow rate, this effluent sulphate concentration was within the severe local legislation norms for surface water of 90 to 150 mg sulphate L^{-1} (norms are for rivers where treated sewage is mostly discharged in; Vlarem, 2010). However, this led to the pH being lower than that allowed for discharge in surface water. Therefore, at an industrial scale, legislation limits on effluent pH and sulphate concentration are more likely to set boundaries on SO_2 removal rates in flue gas-fed microalgal reactors rather than on the toxicity on microalgal species.

4.3.4. Neglected flue gas compounds

The interaction of microalgae with several other flue gas compounds, such as O_2, N_2, H_2O, CO, C_xH_y, particulate matter (PM), halogen acids and heavy metals, have received little attention so far.

Oxygen plays an important role in microalgae reactors for wastewater treatment. It affects not only the chemical oxygen demand (COD) removal from wastewater, but also the NO removal from flue gas (Nagase et al., 2001). Therefore, flue gas might be an interesting source of oxygen. De Godos et al. (2011a) recorded no significant impact of flue gas on the dissolved oxygen (DO) of a microalgae reactor. However, in the latter study, the DO was only measured at daytime (being around saturation) and not at dark. At daytime in undeep microalgae reactors, the O_2 transfer from the flue gas to the reactor liquor is expected to be low due to the relatively low flue gas flow rates used, low oxygen aqueous solubility, low culture depth and the already increased oxygen concentrations in the reactor. Whether oxygen from flue gas can significantly impact the DO concentration in microalgae reactors at night still needs to be examined.

On the other hand, oxygen production by microalgae can become a self-inflicted poison, especially in closed reactors. Indeed, when oxygen accumulates in the culture medium, photorespiration and photo-inhibition can take place, leading to a decrease in biomass yield on light energy (Torzillo et al., 1998). Photorespiration can be avoided by increasing the CO_2:O_2 ratio by adding more inorganic carbon to the reactor, and/or by removing oxygen from the reactor. Several methods exist to remove oxygen from microalgae reactors: (1) mechanical by stirring systems or gas bubbling (Richmond, 2004; Ugwu et al., 2008), (2) photobioreactor design leading to more turbulence or including degassing units (Kumar et al., 2011), (3) biological by addition of organic carbon and using a consortium of both microalgae and bacteria (Van Den Hende et al., 2011b). A recent strategy to decrease photo-inhibition is genetic engineering in which the main focus has been on reducing the size of the chlorophyll antenna (Zeng et al., 2011). In this regard, the effect of flue gas sparging might be greater on O_2 stripping at day, rather than on O_2 supply at night. The O_2:CO_2 ratio of flue gas has been demonstrated to be important. Indeed, lowering this ratio increases the microalgal growth rate (Douskova et al., 2009).

Moreover, if microalgae reactors are used for flue gas polishing to legislation standards, the oxygen concentration of the off-gas plays an important role. Concentrations of flue gas compounds in off gas need to be corrected by a reference O_2 value to prevent dilution of the flue gas by air according to the following formula (EU, 2010):

$E_r = ((21 - O_r).E_m) / (21-O_m)$ (E. 4.38.)

with E_r the corrected concentration (mg Nm^{-3}), E_m the measured concentration (mg Nm^{-3}), O_r the reference oxygen concentration (a fixed percentage according to the burned fuel, for example 3 % for diesel, 6 % for coal), and O_m the measured O_2 concentration in the off-gas (%). In a microalgal reactor, oxygen can be produced leading to an increased oxygen concentration in the off-gas, and thus an increased O_m value and an E_r value higher than one. Therefore, emission standards become more stringent for oxygen producing flue gas treatments. This method is thus not correct for photosynthesis-based gas treatments, as CO_2 is exchanged by O_2 (photosynthetic produced *in situ*) but not diluted by air (containing 21 % O_2). Another standard (e.g., based on N_2) could solve this issue.

N_2 gas, the major component of flue gas, can be fixed by cyanobacteria into ammonia that can then be assimilated (Bothe et al., 2010). This energy-consuming process catalysed by nitrogenase is triggered by low levels of ammonium or nitrate. Moreover, high levels of oxygen, often present in microalgae reactors, can inhibit the activity of nitrogenase (Graham and Wilcox, 2000). Therefore, nitrogen fixation occurs in adjacent heterocysts cells in most microalgal species (Fig. 4.4.) or separated in time and location in non-heterocystous forms. A possible application of N_2 fixation from flue gas could be the treatment of wastewater rich in COD and low in nitrogen by a consortium of cyanobacteria and bacteria.

Water is a component used in photosynthesis. The addition of water due to flue gas injection into the microalgal reactor is expected to be neglectable. This is because prior to this injection, the gas should be cooled and the water vapour condensed to avoid corrosion to the fan.

Direct uptake of CO by microalgae has, to the best of our knowledge, not yet been shown. By contrast, certain microalgae emit CO, and CO oxidation (presumably bacterial) has been observed for living algal cultures during incubations with exogenous CO at concentrations of 125 mg Nm^{-3} (King, 2001). This suggests that bacteria-algae associations might lower CO fluxes in flue gas-sparged non-axenic microalgae reactors. Carboxidotrophic bacteria convert CO into biomass and CO_2 (King and Weber, 2007) and microalgae can use this CO_2 while providing O_2.

Whereas the direct removal of C_xH_y by microalgae remains unknown, its production has been reported. For example, cyanobacteria can produce alkanes (C_xH_{2x+2}), such as tridecane, pentadecane and heptadecane, and alkenes (C_nH_{2n}), such as pentadecene and heptadecene (Schirmer et al., 2010). Indirect removal of CH_4 has been shown to be successful at labscale in microalgal polycultures by a partnership with methanotrophic bacteria (Van der Ha et al., 2011).

The influence of particulate matter on the productivity of microalgae has been investigated for *Nannochloropsis salina* and *Phaeodactylum tricornutum* (Matsumoto et al., 1997). When the medium concentrations of dissolved Ni and V due to PM exceed more than 1 and 0.1 mg L^{-1}, respectively, microalgal productivity decreases.

Hydrogen chloride will rapidly dissociate in an aqueous environment and its toxic effects on phytoplankton are thought to be a result of a pH decrease rather than the effects of the hydrochloric acid itself (UNEP, 2011). Being a strong hydrogen bonding agent, fluoride has the ability to interact with most cellular components and thus shows a multipronged effect on algal cell metabolism. The threshold fluoride concentration at which toxicity manifests varies between eukaryotic microalgae and cyanobacteria, with cyanobacteria being more sensitive, and is strongly pH dependent (Bhatnagar and Bhatnagar, 2000). The toxicity of bromide and iodide on eukaryotic microalgae and cyanobacteria are in the same order, showing toxicity from around 2 g L^{-1} and being orders of magnitude less toxic than certain heavy metals such as cadmium (Flury and Papritz, 1993).

Although traces of heavy metals are essential as co-factors for many enzymatic processes in microalgae, higher concentrations are toxic (Becker, 2008). Mercury is especially detrimental for microalgae in reactors sparged with untreated flue gas (Douskova et al., 2009). Microalgae possess very high metal uptake capacities (uptake and adsorption) and can detoxify a wide range of heavy metals: cadmium, lead, caesium, chromium, cobalt, copper, gold, ferrous, lead, manganese, mercury, nickel, zinc and uranium (de-Bashan and Bashan, 2010). Their use for heavy metal scavenging, alone, immobilised or in concert with bacteria, has been reviewed recently (de-Bashan and Bashan, 2010; Muñoz et al., 2006; Perales-Vela et al., 2006) and therefore, will not be discussed further in this review.

In conclusion, the importance of considering the latter neglected flue gas compounds if sparged in microalgae cultures will strongly depend on the concentration present in the used gas and on the aim of the microalgae cultivation. For example, heavy metals might be present in concentrations toxic for microalgae or limiting the possibilities for biomass valorisation, whereas the concentrations of bromides are generally too low to become toxic for microalgae and therefore can be neglected. If wastewater treatment is the aim, some other neglected flue gas compounds, such as O_2 and N_2, might be in theory a useful resource, but its practical and economic feasibility remains to be investigated.

4.4. Outlook and opportunities

Based on the knowledge reviewed above, novel opportunities for innovative applications and the research needs concerning flue gas-fed microalgal biotechnology are highlighted in this section.

4.4.1. Innovative biotechnological opportunities

Combining flue gas and microalgae in innovative ways opens up pathways for the valorisation of microalgal biomass of which the following applications can be of great interest:

(1) Production of enzymes for environmental technology

Although microalgae may play a key role in making wastewater treatment systems more sustainable, the valorisation of the produced biomass is limited to low-value products such as bio-energy. The production of microalgal enzymes that can catalyse flue gas treatment processes may provide an interesting added value supplied by the microalgal biomass. The potential of CA for enzymatic acceleration of CO_2 capture from flue gas has been demonstrated (Collett et al., 2011). Challenges lie in the poor stability and activity of naturally derived CA in the harsch process conditions of flue gas scrubbing, i.e., high temperatures and/or high pressure, trace contaminants such as heavy metals, as well as NO_x and SO_x (Saville and Lalonde, 2011). Approaches to overcome these limitations have included using strain or protein engineering techniques to create thermo-tolerant enzymes, immobilizing the enzyme for stabilization and confinement to cooler regions, and process modifications that minimize the stress on the enzyme (Saville and Lalonde, 2011). Furthermore, CA

derived from thermophilic microalgae may represent a source of thermostable CAs, similar to its production by the thermophilic Archaeum *Methanosarcina* sp. (Ferry, 2010). A direct application could be the use of microalgal effluent containing extracellular CA to dilute the solvent needed in wet CO_2 gas scrubbers.

Other microalgal enzymes of interest are iron siderophores. Apart from being suggested earlier that microalgal iron siderophores may act as biochelators in enhancing NO_x removal similar to that of Fe-EDTA used in denox processes (Van Den Hende et al., 2011a), the activity of microalgal siderophores as denox catalysts has not been proven yet. Siderophore-mediated iron uptake is of great importance particularly for N_2 fixing cyanobacteria, since they need it for nitrogenase production (Simpson and Neilands, 1976). Whether the sequenced addition of iron to diazotrophic cyanobacteria can enhance NO removal by enhanced production of iron siderophores is yet to be explored.

(2) Enhancing gas production by microalgae

In this study, we also envision the possibility of using flue gas to enhance (i) microalgal production of hydrogen, (ii) methane production of microalgal biomass via anaerobic digestion, and (iii) production of novel biogases. It is well known that sulphur depletion might lead to H_2 production by microalgae (Brennan and Owende, 2010). One approach is the sequencing of sulphate-sufficient and depleted conditions. The use of flue gas containing SO_2 is not only a cheap source of sulphur, but might ease the practical application of this sequencing. Another approach is adding heat-stable carbonic anhydrases to an anaerobic digestor (Borchert and Saunders, 2011). The idea is to convert more CO_2 from the gas phase into bicarbonate, the substrate of methanogenic Archaea, and thereby increase the methane content of biogas. The authors predict a more cost-effective possibility of adding CA-containing microalgal biomass directly to an anaerobic digestor. This could be an extra added value for the low-value microalgal biomass produced in sewage.

Another novel pathway is the direct cyanobacterial production of alkanes, the major constituents of diesel and gasoline (Schirmer, 2010). The ability of cyanobacteria to selectively convert renewable carbohydrate into fuel-grade alkanes without hydrogenation is an important step towards the goal of low-cost renewable transportation fuels, and therefore merits further exploration.

(3) Enhanced removal of carbon and sulphur compounds

As discussed above, microalgae can produce minerals via calcification. Sequenced sparging of flue gas in calcium-rich wastewater is another way of enhancing the biosequestration of CO_2 while producing precipitated $CaCO_3$, a product interesting for the environmental technology sector. Microalgal CA enhances the calcification process (Li et al., 2010). The production of CA and $CaCO_3$ are intertwined, and could thus be combined.

By partnerships with bacteria, CO and C_xH_y can also be removed in flue gas-sparged microalgae reactors. While bacteria convert CO or C_xH_y into CO_2 using O_2, microalgae provide this O_2 and take up CO_2 via photosynthesis. The proof-of-principle has been shown for methanotrophic bacteria combined with microalgae (van der Ha et al., 2011), but the application with CO and other C_xH_y from flue gas remains to be seen.

If the aims are enhanced removal of SO_2 and concomitant SO_2 trade revenues, one should focus on microalgal species possessing very specific ways of storing sulphate. For example, the cell wall-lacking red algae *Porhyridium* sp. can store sulphur as sulphated polysaccharides in its mucilaginous matrix, several radiolarians can store it in crystalline strontium sulphate shells, while the *Closterium* sp. can store it as barium sulphate crystals in vacuoles of its cell tips (Graham and Wilcox, 2000). This suggests that some opportunities are available to enhance sulphur scavenging from flue gas, thereby protecting species more sensitive to high sulphur concentrations in a mixed bacteria/microalgae consortium.

(4) Steering the biochemical composition and biomass availability

Flue gas can be used to steer the biochemical composition of microalgae. CO_2 present in this gas affects the contents of lipid and fatty acids, proteins, polysaccharides and chlorophyll (Table 4.3.).

The resistance of microalgal cell walls is one of the main bottlenecks for anaerobic digestion of microalgae (Sialve et al., 2009). Sparging with toxic flue gas compounds to disrupt microalgal cell walls may be an interesting pre-treatment of microalgal biomass for anaerobic digestion, if an optimal pH can be maintained.

(5) Cultivation of extreme acidophilic species

Up to now, less than 50 microalgad species have been screened for their tolerance to certain flue gas compounds (Table 4.5.). Screening more species for their

tolerance to high loading rates of acid flue gas compounds, especially acidophilic species, is needed. The cultivation of extreme acidophilic species provides an interesting approach in systems where monoculture is the aim.

4.4.2. Needs for research and policy adjustment

Although there has been a plethora of studies on CO_2 removal in microalgae reactors, results are difficult to compare partly due to partial parameterisation. For example, information on the concentrations of flue gas compounds or flue gas flow rates is not available in several papers to calculate the flue gas loading. Moreover, it should be clearly stated whether the assessed CO_2 removal is due to fixation into microalgal biomass or to dilution of CO_2 into the reactor water. This should be a particular point of attention for future publications not only to compare different studies but also to make it possible to translate experimental results to realistic revenues of CO_2 credits.

Furthermore, there are still a lot of fundamental research niches regarding the interaction of flue gas compounds and microalgae that require further investigation. Firstly, closed C-, N- and S-balances in flue gas-fed microalgae reactors, measuring all organic and inorganic compounds of C, N and S in the gas, liquid and biomass, need to be made. Secondly, other compounds such as NO_x, SO_x, C_xH_y, CO, halogen acids and particulate matter warrant further exploration, especially their biochemical uptake and assimilation pathways, and their effects on the biochemical composition of microalgae and enzyme production. The importance of a deeper understanding of these pathways is reflected in the large variety of applications of already well-described CO_2 pathways such as calcification, which can lead to more effective microalgal resource management.

For real progress to continue at industrial scale, process technology should be further optimised to ascertain a basic assimilation of the major flue gas compounds while avoiding toxicity of other compounds and producing biomass for multi-products (biorefinery concept). In this, outdoor reactors at pilot and demonstration scale are needed, especially to make a realistic analyse of the economical and technological potential of various flue gas fed microalgae cultivation systems. Moreover, special attention should be given to provide realistic data on the volumes of flue gas needed/treated in these systems compared to the produced volumes of flue gas.

Besides these needs for academic and industrial research, there are still some themes regarding legislation that need to be clarified by local policy makers:

- the type of microalgae reactors (open or closed) that are allowed for flue gas treatment and its concomitant off-gas discharge standards;
- the method to determine the amount of earned CO_2, NO_x and SO_x credits in flue gas-fed microalgae reactors: removal of these compounds by microalgae or by the whole reactor device including dissolution of the compound in the aqueous phase;
- an adjustment of the O_2 reference method for flue gas treatment in microalgae reactors, since oxygen is produced in these reactors that make current emission norms more difficult to obtain compared to other oxygen using or oxygen neutral treatment systems.

4.5. Conclusions

Flue gases contain several chemical compounds, which even at concentration levels of treated flue gas, can affect the growth, biochemical composition and excretion of microalgae. Vice versa, microalgae can also directly and indirectly affect the removal of flue gas compounds such as CO_2, NO_x, SO_x, heavy metals, and unburned carbohydrates. Several detailed studies on interactions between CO_2 and microalgae have been published. However, for all other compounds, this is nearly unexplored in the scientific literature.

Flue gases are a resource yet to be fully utilised in microalgal biotechnology, not only to moderate the anthropogenic effects on our climate and earn carbon credits, but also to steer microalgal resource management towards innovative applications of microalgal biomass compounds. Whether these carbon credits could significantly lower the algae production cost, depends on the scientific method used to assess CO_2 removal – one of the mentioned items which should be clarified by policy makers. For researchers, there are still a lot of flue gas application niches to be explored amongst others: production of catalysts for environmental technology, enhanced (bio)gas production, enhanced removal of carbon and sulphur compounds, enhanced biomass composition and availability, and culture of extremophiles.

4.6. Acknowledgements

This work was supported by an academic Ph.D. grant from Howest University College West-Flanders and Ghent University. We thank T. Lacoere (UGent) for the very nice graphic design of Figures 4.2, 4.3 and 4.4; P. Dejans (Howest) for the discussions on flue gas treatment; J. Coppens, W. De Muynck and C. Zamalloa (UGent) for critical reviewing this manuscript.

We can't solve problems by using the same kind of thinking as we used when we created them.

(Einstein, 1879-1955)

Background picture:

MaB-floc SBRs treating calcium-rich paper mill wastewater.

CHAPTER 5

Extracellular carbonic anhydrase enhances calcium removal from paper mill UASB effluent by microalgal bacterial flocs

Abstract

Calcium-rich UASB effluent from recycling paper mills results in problematic scale formation. Moreover, treatment in aerated activated sludge reactors results in high aeration costs. Therefore, a novel treatment is proposed for paper mill UASB effluent in microalgal bacterial floc sequencing batch reactors (MaB-floc SBRs) in which oxygen is provided via photosynthetic aeration and calcium is removed via bio-mineralization. Inhibiting extracellular carbonic anhydrase (CA), an enzyme catalysing the hydradation/dehydradation of CO_2, significantly decreased the removal of calcium and inorganic carbon by MaB-flocs at neutral pH. This demonstrates the importance of CA in treatment of paper mill UASB effluent. MaB-flocs contained 10.3 ± 3.2 % calcium and biologically-influenced calcite crystals. Harvesting by 200 µm filter press recovered over 99 % of the MaB-flocs; addressing a major bottleneck of microalgae wastewater treatment. This CA-enhanced calcium removal and efficient MaB-floc harvesting highlight the potential of MaB-floc SBRs treating calcium-rich wastewater.

Chapter redrafted after:

Van Den Hende, S., Rodrigues, A., Hamaekers, H., Boon, N., Vervaeren, H. Extracellular carbonic anhydrase enhances calcium removal from paper mill UASB effluent by microalgal bacterial flocs. Submitted.

5.1. Introduction

In paper production, water serves as an agent for suspension and transport of fibres and fillers, as a solvent for chemical additives and as a medium to build hydrogen bridge bonds between fibres (Möbius, 2006). For the production of one ton of paper, 35-1000 m^3 process water is needed (Möbius, 2006; Thompson *et al.*, 2001). To decrease this high water use, paper mills reuse treated wastewater resulting in an increased amount of organic carbon and calcium hardness in the process water (Kim et al., 2002). The organic carbon in the process water, if more than 2 g chemical oxygen demand (COD) L^{-1} (Möbius, 2006), can be valorised in a biogas producing upflow anaerobic sludge blanket (UASB). In this way, lower operational and capital costs can be obtained compared to a sole aerated activated sludge (AAS) system (Lerner et al., 2007). Nevertheless, UASB effluent never has a sufficient quality of Biological Oxygen Demand (BOD) and COD for direct discharge or reuse. Therefore, generally this UASB is followed by an AAS (Lerner et al., 2007) leading to high energy costs due to mechanical aeration (up to 50 %) (Tchobanoglous et al., 2003).

In addition to organic carbon, paper mill UASB effluent contains a high concentration of calcium (e.g., 345-793 Ca^{2+} mg L^{-1}) and alkalinity (e.g., 464-1453 mg $CaCO_3$ L^{-1}) (Kim et al., 2002). This is of practical concern because it leads to accelerated scale formation in paper mill pipelines and nozzles and is one of the major factors to force paper mills not to reduce water consumption (Boyko et al., 1999). Current chemical processes for calcium removal from paper mill wastewater are not economically feasible because they require caustic soda and lime (Mauchauffée et al., 2012). Moreover, this chemical treatment might lead to an increased conductivity and decrease the efficiency of additives used in paper mills (Hulkko and Deng, 1998).

Gravity settling microalgal bacterial flocs in sequencing batch reactors (MaB-floc SBRs) treat wastewater based on photosynthetic aeration without any need of mechanical aeration (Van Den Hende et al., 2011a). MaB-flocs contain, next to bacteria, microalgae and cyanobacteria (further referred to as microalgae, to ease reading). These microorganisms may create microenvironments enhancing calcite precipitation in calcium-rich wastewater via several microbial mechanisms (Fig. 5.1.). The calcium concentration of paper mill UASB effluent can be locally raised by microalgae by the presence of Ca^{2+} binding domains (Fig. 5.1.g) and by the export of Ca^{2+} through the Ca^{2+}/H^+ translocator (Fig. 5.1.p) (Jansson and Northen, 2010).

Moreover, the inorganic carbon level of wastewater can be increased via bacterial and microalgal respiration in MaB-flocs (Van Den Hende et al., 2011a), and by carbon concentrating mechanism of microalgae (Fig. 5.1.q) (Jansson and Northen, 2010). Besides, microalgae can produce extracellular polymeric substances (EPS) facilitating calcite nucleation (Fig. 5.1.r) (Dupraz et al., 2009). During oxygenic photosynthesis, part of the solar energy can be dissipated as heat (Richmond, 2004) and increase the calcium carbonate saturation state (Jensen, 2003). Besides, photosynthetic growth increases the pH (Fig. 5.1.a) which shifts the carbon species more towards bicarbonate and carbonate (Fig. 5.1.c) (Jansson and Northen, 2010). Carbonic anhydrase (CA), a metalloenzyme catalysing the hydration and dehydration of CO_2, can accelerate calcite precipitation (Fig. 1d) (Kim et al., 2012). Certain microalgae excrete CA, especially in CO_2 deplete conditions (Van Den Hende et al., 2012a). Extracellular CA plays a key role in calcification in natural environments (Kuprianova et al., 2007).

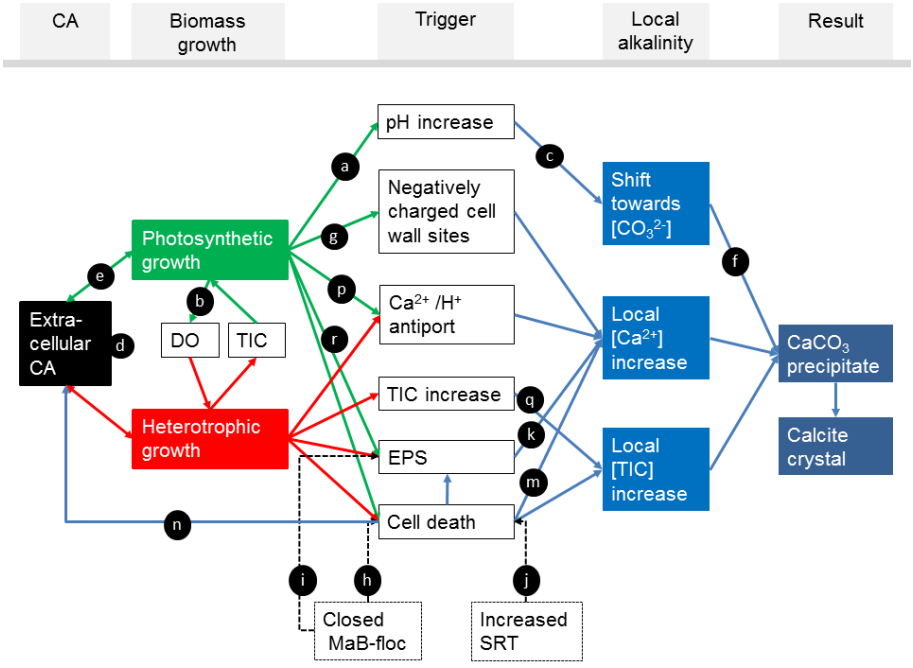


Fig. 5.1. *Proposed models for interplay of microbial mechanisms enhancing $CaCO_3$ precipitation in MaB-floc reactors treating paper mill UASB effluent*
——► : Adapted from microbial mechanisms presented by Jansson and Northen, 2012; Van Den Hende et al., 2012; Dupraz et al., 2009; - - - - ►: hypothesis based on this study; single letters refer to the text.

All the above outlined microbial mechanisms may enhance calcite precipitation in MaB-floc reactors treating paper mill UASB effluent, and in this way increase the calcium and carbon removal. However, the presence of extracellular CA and its effect on calcium removal in MaB-floc SBRs treating industrial wastewater hasn't been shown yet. Besides, the pH increase by photosynthesis in MaB-floc SBRs, which is beneficial for calcite precipitation, needs to be balanced to allow reuse and/or discharge of the treated paper mill wastewater (pH lower than 9; Vlarem II, 1995). Therefore, an optimal pH for reactor operation needs to be found.

In this study, the potential of MaB-floc SBRs to treat paper mill UASB effluent was investigated as an alternative for AAS reactors (Fig. 5.2.a; Fig. 5.2.b), through focusing on calcium and carbon removal. To assess the hydraulic retention time (HRT) and initial pH of SBR cycles, reactors were first operated in batch mode; mimicking one SBR cycle starting with an initial neutral pH (7) and increased pH (8). The presence of extracellular CA and its effect on the reactor performance was investigated. To further screen the industrial feasibility of MaB-floc SBRs for treatment of paper mill UASB effluent, the effluent quality in terms of pH, TN and TP and a single-pass filter press to efficiently harvest MaB-flocs were evaluated.

5.2. Materials and methods

5.2.1. Set-up of the batch experiments

MaB-flocs for the batch experiments were pre-cultured in sequencing batch reactors (SBRs) of 4 L as described by Van Den Hende et al. (2011) with a consortium of microorganisms collected at a paper mill (Stora Enso, Ghent, Belgium): (1) local photosynthetic microorganisms from wastewater treatment tanks predominantly containing microalgae (*Desmodesmus* sp., unidentified coccal microalgal sp.), diatoms (*Nitzchia* sp.), and cyanobacteria (*Phormidium* sp.; *Oscillatoria* sp.) (microscopic determination) and (2) aerobic activated sludge. Reactors were fed with paper mill wastewater collected just after the urea and phosphate addition step and before the aerobic activated sludge tank (Stora Enso, Ghent, Belgium).

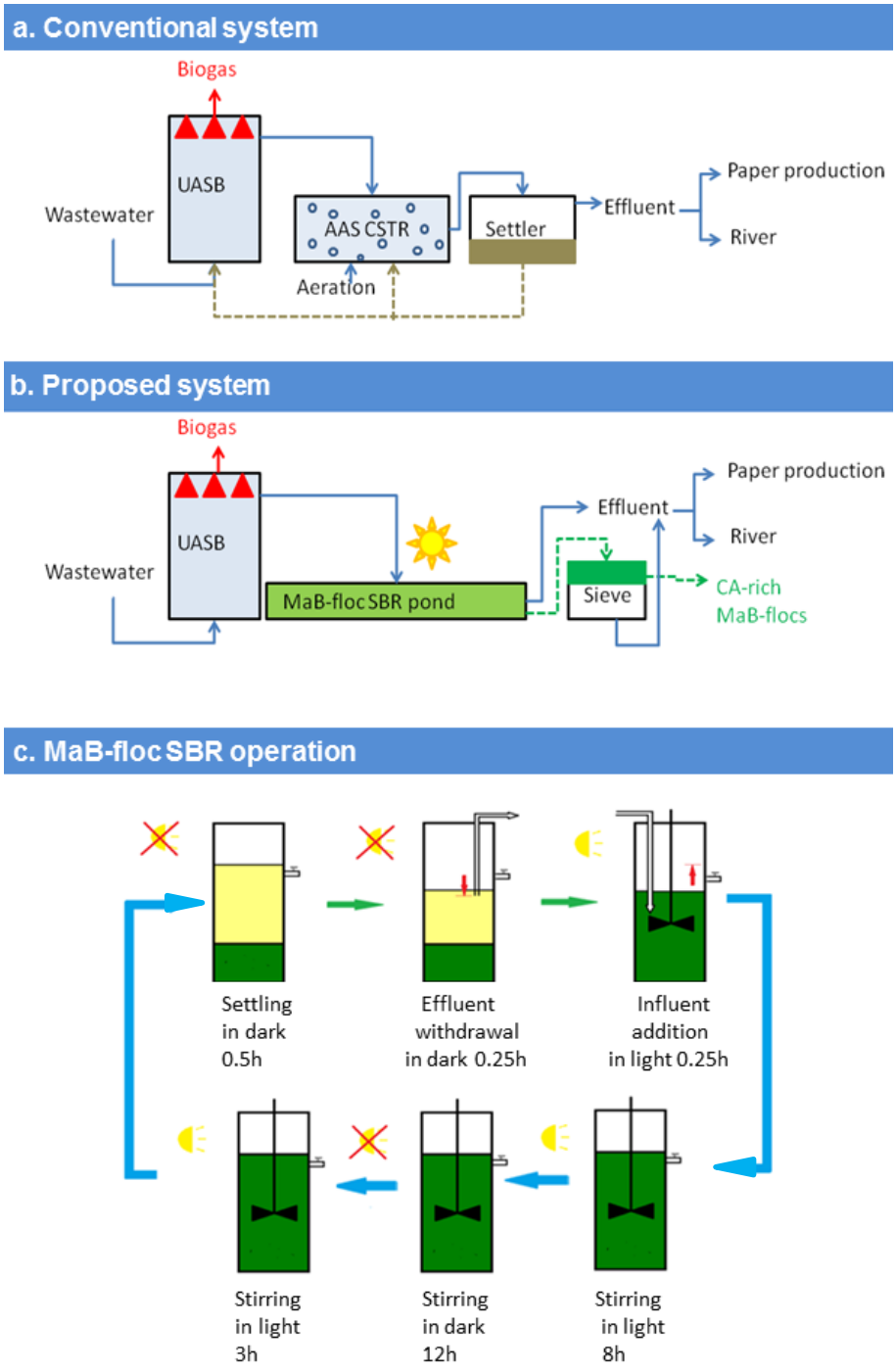


Fig. 5.2. *Conventional (a) and proposed (b) processes for treatment of recycled paper mill wastewater, and operation of lab-scale MaB-floc SBRs treating paper mill UASB effluent (c)*

Batch experiments were performed in closed tubular photobioreactors of 5 L with a working volume of 4 L as previously described (Chapter 3). For each reactor, one halogen lamp (EcoHalo R7S 8700 lum 400W, Philips, Belgium) provided an average photon flux density (PFD) of 122 µmol PAR photons m^{-2} s^{-1} (Li-COR LI-250A, USA) at the inner reactor wall with a 12 h light:12 h dark cycle. The reactors were stirred continuously (120 rpm; RZR2020, Heidolph, Germany). No additional aeration was performed, restricting oxygen supply to *in situ* biological oxygen production. Dissolved oxygen (DO) (HI8410, Hanna Instruments, Germany), pH and temperature (T) (Ecotrans pH03, Jumo, Belgium) were continuously logged.

To assess the HRT and initial pH for further SBR operation, MaB-floc reactors were operated as batch mimicking one SBR cycle from influent feeding till effluent withdrawal. Since photosynthetic activity by MaB-flocs increases the reactor pH (Van Den Hende et al., 2011a), increasing the HRT will increase the initial pH of an SBR cycle. Therefore, two batch reactor set-ups were compared: B_{neut} in case of a low HRT and thus starting with a neutral pH, and B_{high} in case of an increased HRT and thus starting with a high pH which was increased by photosynthesis. Three times a day (at the end of the dark phase, 5 h after starting the light phase, at the end of the light phase), 0.075 L of reactor liquor was sampled. To mimick the settling phase and effluent withdrawal of an SBR cycle, 0.030 L of this sample was settled for 30 min (as in MaB-floc SBR operation, Van Den Hende et al., 2012a) and its supernatant was analysed. To investigate the effect of extracellular CA, parallel batch reactors ($B_{neut+CAinhib}$ and $B_{high+CAinhib}$) were set up with influent containing 1mM acetazolamide (AA) (A6011, Sigma, USA) which inhibits extracellular CA but does not penetrate the cell (Moroney et al., 1985).

All batch reactors (Table 5.1.) were started up with 2 L MaB-floc inoculum (containing 4.00 g MaB-flocs TSS) and 2 L paper mill wastewater (Stora Enso, Ghent, Belgium) supplemented with Ca-$CaSO_4.2H_2O$ (50 %w/w) (Ferak Laborat, Germany) and Ca-$CaCl_2$ (50 %w/w) (Prolabo VWR, Belgium) to 500 mg Ca^{2+} L^{-1}. To assess the calcium removal without MaB-flocs, a blank experimental run ($B_{neut\ control}$) was performed with 2 L calcium enriched paper mill wastewater and 2 L of MaB-flocs supernatant without MaB-flocs (Table 5.1.). Reactor liquid was analysed for total suspended solids (TSS), volatile suspended solids (VSS) and calcium content of VSS and TSS, and microscopic observations were performed. Total calcium (TCa), total

inorganic carbon (TIC), total organic carbon (TOC) and total carbon (TC) were determined of the settled supernatant.

Table 5.1. *Overview of the different reactor set-ups used in this study*

Reactor	Type	MaB -flocs	Initial pH[a]	Concentration CA-inhibitor (mM AA)[b]	Operation period (days)
$B_{control}$	Batch	No	Neutral	0	9
B_{neut}	Batch	Yes	Neutral	1	9
$B_{neut+CAinhib}$	Batch	Yes	Neutral	1	9
B_{high}	Batch	Yes	High	0	9
$B_{high+CAinhib}$	Batch	Yes	High	1	9
SBR	SBR	Yes	Neutral	0	50
$SBR_{+CAinhib}$	SBR	Yes	Neutral	1	50

[a] pH at the start-up of a batch reactor experiment or at the start of a SBR cycle just after influent addition;
[b] Acetazolamide (AA) which inhibits extracellular carbonic anhydrase (CA).

5.2.2. Set-up of semi-continuous experiments

MaB-flocs for the semi-continuous experiments were pre-cultured similar as in the batch experiments, starting from microorganisms from a water recycling paper mill with UASB (VPK, Dendermonde, Belgium): (1) local photosynthetic microorganisms collected from wastewater tank walls predominantly containing microalgae (unidentified coccal microalgal sp.; *Spirogyra* sp.), next to diatoms (*Nitzchia* sp.), and cyanobacteria (*Phormidium* sp.) (microscopy observations), and (2) aerobic activated sludge. SBRs were fed with thermophile UASB effluent from this paper mill stored at 4°C until use.

For the semi-continuous experiments, the same reactors were used as in the batch experiments, but these were operated in SBR modus (Fig. 5.2.c). Peristaltic pumps were used for effluent withdrawal (Watson Marlow, USA) and diaphragm pumps (Blackstone, USA) for influent feeding. SBRs were operated at a HRT of 4 days. No additional aeration was performed. Feeding, stirring and decanting were controlled by a PLC; irradiation by mechanical timers. Two SBR set-ups were operated: SBR and $SBR_{+CAinhib}$ (Table 5.1.). To preserve AA-activity, 2 times a week 10 L of influent for $SBR_{+CAinhib}$ was prepared by adding 1 mM AA and was stored at 4 °C. Three times a week, reactor liquor was harvested 15 minutes before the settling

phase to maintain the MaB-floc density. A part of this biomass was used for TSS, VSS, chlorophyll *a* (Chl*a*) content, calcium content, calcite content, dewatering and microscopy. Influent and effluent were analysed three times a week for TC, TIC, TOC, TCa, dissolved calcium (DisCa) and pH. Samples of influent, effluent and centrifuged (3750 xg) MaB-floc pellets were stored at -18 °C for CA-analyses.

5.2.3. Analytical methods

Wastewater characteristics

TC, TIC, TOC, TN and TP were measured spectrophotometrically (Hach Lange test kits; HL DR 2800, Belgium). Calcium was determined by ICP-OES (396.847 nm; Vista-MPX, Varian, Australia) on acidified samples (1 % v/v HNO_3) (APHA et al., 2005). DisCa was analysed after filtering at 5-30 µm (VWR, Belgium) and at 0.20 µm (pore size syringe filter RC-20125, Chromafil, Germany). The calcium carbonate saturation state (Ω_{CaCO3}) was calculated from calcium and carbonate concentrations at the measured in situ temperature (T) and pH as:

$$\Omega_{CaCO3} = [Ca^{2+}][CO_3^{2-}]_{T,\,pH} / K_{sp} \qquad \text{(E. 5.1.)}$$

with $[CO_3^{2-}]_{T,\,pH} = [TIC] / (1 + ([H^+] / K_2) + ([H^+]^2 / (K_2 * K_1)))$

with K_{sp} the equilibrium solubility product of calcite of $10^{-8.48}$, and K_1 the first dissociation constant and K_2 the second dissociation constant for carbonic acid for each specific T (Jensen, 2003).

MaB-floc characteristics and harvesting

TSS, VSS and Chl*a* of MaB-flocs were determined as previously described (Chapter 2). To harvest MaB-flocs, reactor liquor (250 mL) was put on a net with pore size of around 200 µm (Euroshop, Belgium), and an external pressure of 40.2 kPa was applied on the MaB-flocs during 5 minutes. The dry matter content of the harvested flocs was determined by drying overnight at 100 ºC. To determine the MaB-floc recovery by harvesting, the Chl*a* content of the filtrate was related to the Chl*a* content of TSS of the SBR liquor.

Calcium and calcite in MaB-flocs

MaB-floc reactor liquor was vacuum filtered at 0.45 µm (Porafil 47 mm, Germany), dried at 100 °C and ashed at 550 °C. Ashed MaB-flocs were dissolved in 1 %v/v HNO_3 and the calcium content determined with ICP (396.847 nm). Mineralogical composition of MaB-flocs was determined with X-Ray Diffraction

(XRD). At the end of the SBR run, MaB-flocs were harvested, dehydrated with 30 % v/v ethanol, dried at 40 °C for 48 h, crushed in a porcelain mortar and sieved at 250 µm. In an agate mortar, 2.7 g of sample was mixed with 0.3 g ZnO internal standard, sieved at 250 µm and loaded in a holder for XRD analysis (Philips PW 1830 diffractometer at 45 kV and 30 mA, Cu-Kα-radiation, 5-65 2θ, stepsize 0.02 °; 2 s scan speed). The obtained diffraction patterns were analysed by DIFFRAC^{plus} EVA software (Bruker AXS). Microscopic observations were performed (optical microscope, Reichert Neovar 300422, Austria). Image acquisition was done on an Axioskop 2 Plus bright field and epifluorescence microscope (Zeiss, Oberkochen, Germany) on fresh samples and SEM (JSM–6400–SEM, Jeol, Japan; 10 kV acceleration voltage; probe current of 8) on liquor samples dried on glass fibre membranes (Porafil, Germany) in silica gel desiccators and gold coated with a sputter coater (Biorad CA508, USA).

Carbonic anhydrase

CA was separated by gel electrophoresis (65 min, 200V) under non-reducing conditions using a molecular mass standards containing CA of 31kDa (161-0317, Biorad, USA). MaB-floc samples were washed twice and sonicated in an ice-batch (5 min, cycle time 0.7, amplitude 70 %, dr. Hielscher Ultrasonic Processor UP 400 S 400 W 24kHz, Germany). MaB-floc and wastewater samples were centrifuged (3750 xg, 5 min), supernatants treated with 0.15 M NaCl and 0.125 M Tris-HCl buffer solution, heated (5 min at 100 °C), cooled (15 min at -18 °C), shaken (1 h) and 10-30 µL loaded on an SDS-PAGE gel (120 g L^{-1}; Biorad, USA).

5.2.4. Statistical analyses

Statistical analyses were performed using PASW Statistics 21 (SPSS Incorporated, Chicago, Illinois). Normality of the data and homogeneity of variances were determined with a Kolmogorov Smirnov test and Levene's test respectively. Where normal distribution was observed, the differences in means were analysed with one-way ANOVA and Tukey's post-hoc test ($p < 0.05$). Where no normal distribution was observed, the differences in means were analysed by Kruskal Wallis including a Bonferroni correction ($p < 0.05$). Means and standard deviations are given.

5.3. Results and discussion

5.3.1. Batch experiments

5.3.1.1. Initial neutral pH

Batch reactor experiments demonstrated the proof-of-principle of removal of TCa, TOC and TIC by MaB-flocs with an initial neutral pH (Fig. 5.3.). For example, after 4 days 46.4 % of TCa (Fig. 5.3.a), 81.9 % of TOC and 61.3% of TIC (Fig. 5.3.e) were removed from the supernatant in B_{neut}. In the control batch reactor without MaB-flocs ($B_{control}$) only 8.7 % TCa (Fig. 5.3.a) and 52.7 % of TOC was removed, while TIC was increased with 35.8 % (Fig. 5.3.e).

The calcium removal was clearly influenced by the dark:light phases of the MaB-floc batch reactor, suggesting a key role of photosynthesis in calcium removal. For example, during the light phase of day 4 in B_{neut}, 30.9 mg TCa L^{-1} was removed (Fig. 5.3.a) while the pH increased from 6.92 to 8.21 (B_{neut}; Fig. 5.3.b). During the following dark phase, only 5.1 mg TCa L^{-1} was released from the settling MaB-flocs to the supernatant while the pH steeply decreased to 7.00 (B_{neut}; Fig. 5.3.b). This is even lower than the initial reactor pH of 7.27. This shows that during light phases, MaB-flocs removed calcium in such a way that it was not released again to the supernatant by a decreasing pH during dark. The same trend was observed in all other reactor operation days until day 4, after which a maximum calcium removal was reached (B_{neut}; Fig. 5.3.a, 5.3.b). Crystals present inside the MaB-floc, but not outside the MaB-flocs in the surrounding water (microscopic observations), suggest that Ca could have been trapped via mineralization into calcite crystals which hardly dissolve. Photosynthetic aeration in the MaB-floc SBR was clearly demonstrated. Indeed, the DO in B_{neut} increased during light phase to supersaturated concentrations of up to 20 mg L^{-1} without mechanical aeration. This was much higher compared to $B_{neut\ control}$ (Fig. 5.3.c). During dark phase the DO decreased to zero (Fig. 5.3.c).

To screen the effect of extracellular carbonic anhydrase, acetazolamide was added, a specific, high-affinity inhibitor of extracellular carbonic anhydrase (CA) (Moroney et al., 1985). Inhibiting extracellular CA in $B_{neut+CAinhib}$, decreased the removal of TCa from 46.4 % to 30.9 %, of TIC from 61.3 % to 46.4 %, and of TOC from 81.9 to 59.7 % after 4 days (Fig. 5.3.a; Fig. 5.3.e). This inhibition decreased the diurnal fluctuations of pH (Fig. 5.3.b) and DO (Fig. 5.3.c). These results suggest the presence of extracellular CA in B_{neut}. Moreover, it suggests that CA not only plays a

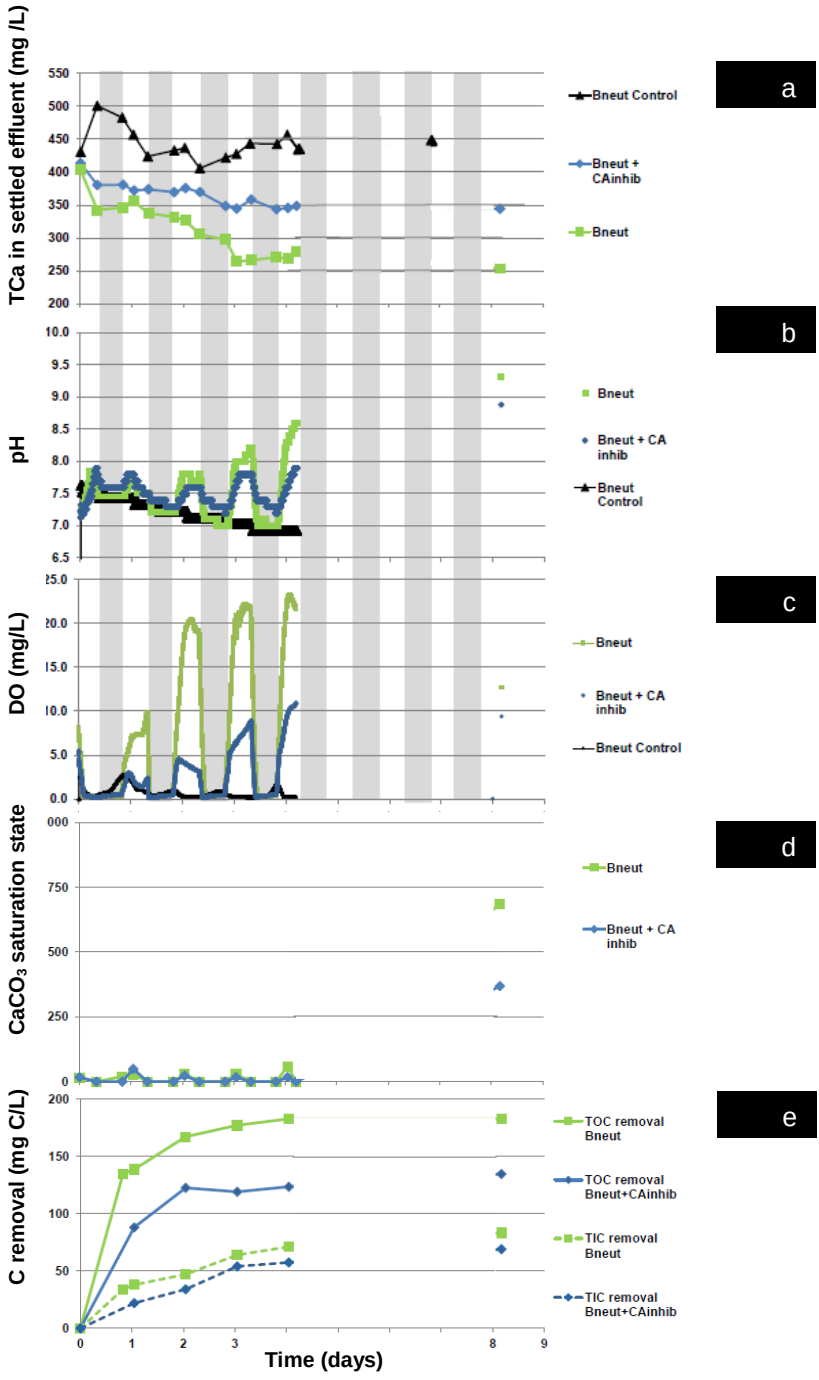
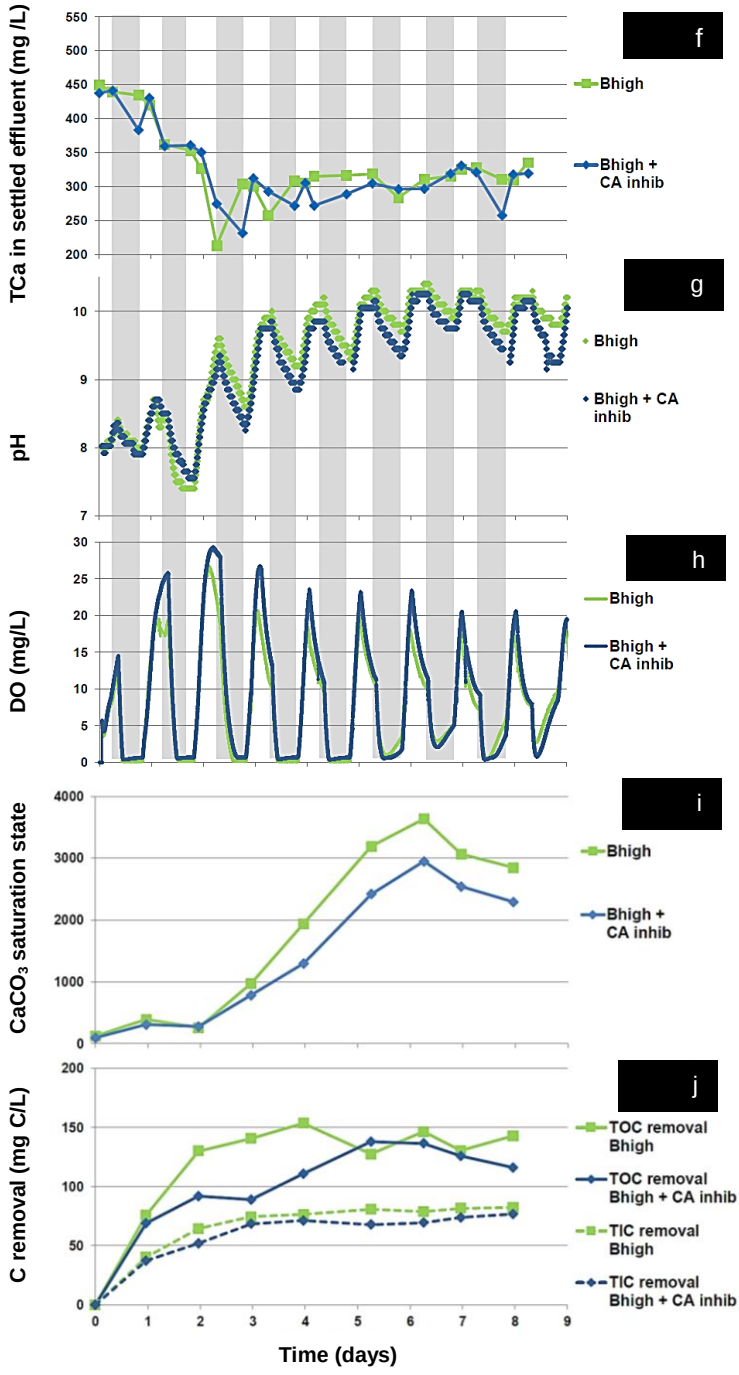


Fig. 5.3. *Effect of inhibiting extracellular CA on treatment of paper mill wastewater in MaB-floc batch reactors with initial neutral and high pH: total calcium content of effluent (a, f), reactor pH (b, g), reactor DO (c, h), $CaCO_3$ state Ω_{CaCO3} (d, i), and organic and inorganic carbon removal (e, j)* *(f-j: on next page)*



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role in oxygenic photosynthesis, i.e. uptake of CO_2 which increases the pH. CA can also play a role in respiration, i.e. release of CO_2 which decreases the pH, by MaB-flocs, as earlier reported for several prokaryotes (Smith and Ferry, 2000).

5.3.1.2. Initial high pH

Increasing the initial pH in B_{high} did not result in an increased removal of TCa in B_{high} compared to B_{neut} (Fig. 5.3.f; Fig. 5.3.a). This was observed despite the stronger increase of the reactor pH (Fig. 5.3.; Fig. 5.3.b) and of the calcium carbonate saturation states (Ω_{CaCO3}) in B_{high} compared to B_{neut} (Fig. 5.3.i.; Fig. 5.3.d). For example, after 4 days, a similar amount of TCa was removed in B_{high} (38.9 % TCa; Fig. 5.3.f) compared to B_{neut} (46.4 % TCa; Fig. 5.3.a), while the reactor pH was 9.90 compared to 8.33 (Fig. 5.3.g; Fig. 5.3.b) and the Ω_{CaCO3} of the settled supernatant was 1945 compared to 59 (Fig. 5.3.i; Fig. 5.3.d), in B_{high} and B_{neut} respectively. The final pH in B_{high} (Fig. 5.3.g) reached values higher than the norms for discharge (Vlarem II, 1995) and reuse (Vito et al., 2010). This pH was similar to the pKa of bicarbonate-carbonate equilibrium, referring to an equal presence of carbonate and bicarbonate

The Ω_{CaCO3} values in B_{high} (Fig. 5.3.j) were notably higher than earlier reported ranges necessary for calcification by cyanobacteria (> 6-10) or anoxygenic phototrophic microorganisms (50-100) (Bundeleva et al., 2012, and references therein). This difference in Ω_{CaCO3} did not affect the percentage of dissolved calcium in the supernatants, being 92.2 ± 7.4 % in B_{high} and 96.8 ± 5.5 % in B_{neut}. In B_{high}, the maximum TCa removal was already obtained after 3 day periods (Fig. 5.3.f) compared to 4 day periods in B_{neut} (Fig. 5.3.a). However, this advantage of a faster calcium removal in B_{high} is offset by the increased effluent pH in this reactor. Indeed, an increased pH is disadvantageous for water reuse and discharge (Vlarem II, 1995).

In contrast to B_{neut}, inhibiting extracellular CA did not lower the TCa removal in $B_{high+CAinhib}$ (Fig. 5.3.f, 5.3.a). This might be explained by the fact that at a pH higher than 8.3, as in B_{high}, carbonate is present and can be directly converted to calcium carbonate without the need for CA (Jansson and Northen, 2010):

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \qquad \text{(E. 5.2.)}$$

Moreover, at a pH above 8.3, the hydroxylation of CO_2 to bicarbonate and further conversion to carbonate is a pathway which doesn't involve the by CA catalysed dehydration and hydration of CO_2 (Wang et al., 2010):

$CO_2 + OH^-$ <-> HCO_3^- (E. 5.3.)

This could be another explanation why the addition of CA inhibitor in $B_{high+CA}$ did not lead to a decreased calcium removal compared to B_{high}. In contrast, at a pH lower than 8.3, as in B_{neut}, $CaCO_3$ is mainly produced by conversion of bicarbonate to CO_2 and carbonate (Jansson and Northen, 2010):

$Ca^{2+} + 2\ HCO_3^-$ -> $CaCO_3 + CO_2 + H_2O$ (E.5.4.)

The CO_2 release in this equation (E.5.4.) at a pH below 8.3 is presumably enhanced by CA catalyzing the hydration and rehydration of CO_2. It could explain why inhibiting extracellular CA affected the by photosynthesis and respiration driven pH fluctuations at neutral pH but not at high pH (Fig. 5.3.c, 5.3.g).

The slightly decreased TOC removal in $B_{high+CAinhib}$ compared to B_{high} (Fig. 5.3.j) and thus slightly decreased TOC oxidation and oxygen consumption, can explain the slightly higher DO in $B_{high+CAinhib}$ compared to B_{high} (Fig. 5.3.h). After 5 days, the TOC removal in $B_{high+CAinhib}$ stabilized to similar values as in B_{high}. Overall, these results suggest that extracellular CA does not play a key role or that its concentration or activity is very low in MaB-floc batch reactors with initial high pH.

5.3.1.3. Conclusions for SBR operation

MaB-floc SBRs treating paper mill UASB effluent should be operated in such a way so that each SBR cycle starts with a neutral pH, because this leads to a less basic effluent pH. It is important that the effluent is not too high, i.e. not higher than the standards for or reuse (Vito et al., 2010) or discharge (Vlarem II, 1995). Besides, only at initial neutral pH, extracellular CA increased the calcium removal. Moreover, the maximum calcium removal was similar with an initial neutral pH compared to an initial high pH. SBR operation at an initial high pH would be disadvantageous because it entails increasing the pH via one or both of the following strategies. First, a strong pH increase via photosynthesis, means an increased HRT. This results in increased reactor dimensions and thus treatment costs. Second, a pH increase by chemical addition brings additional cost for chemicals and causes higher conductivity in process water. The latter lowers the efficiency of additives used in paper mills (Mauchauffée et al., 2012; Hulkko and Deng, 1998).

Since the removal efficiencies of TCa, TIC and TOC in B_{neut} stabilised after 4 days, a HRT of 4 days seems sufficient for a MaB-floc SBR. Effluent withdrawal

and influent addition of the SBR should be done after a light phase, to aim at aerobic conditions during influent addition avoiding denitrification. Furthermore, to obtain a dischargeable effluent pH, this light phase should be short and effluent withdrawal should take place daily. Moreover, this light phase should be short so a long enough light phase remains after influent addition. In this way, sufficient TOC oxidation is enabled and anoxic or anaerobic conditions during the dark phase avoided.

5.3.2. Semi-continuous experiments

5.3.2.1. Calcium removal and calcite precipitation

Calcium was significantly removed from paper mill UASB effluent in the MaB-floc SBR (Table 5.2.). This calcium removal was influenced by several microbial mechanisms (Fig. 5.1.).

Photosynthetic pH increase (Fig. 5.1.a) and aeration (Fig. 5.1.b) were demonstrated by the diurnal fluctuations of pH and DO in the MaB-floc SBR (Fig. 5.4.a; Fig. 5.4.b). This photosynthesis led to an increased effluent pH of SBR (Table 5.2.) and shifted the carbon species more towards carbonate (Fig. 5.1.c). This increased the average Ω_{CaCO3} of the effluent 8 times compared to the influent (Fig. 5.4.c), being 24.2 ± 8.7 in the influent and 191 ± 63 in the effluent.

Inhibiting extracellular CA significantly decreased the average removal of TCa in $SBR_{+CAinhib}$ compared to SBR (Table 5.2.). This suggests a key role of extracellular CA in calcium removal in MaB-flocs SBRs treating paper mill UASB effluent (Fig. 5.1.d). Compared to SBR, $SBR_{+CAinhib}$ showed a significantly decreased TIC removal (Table 5.2.), decreased diurnal pH fluctuations (Fig. 5.4.b), significantly decreased effluent pH (Table 5.2.) and decreased Ω_{CaCO3} (Fig. 5.4.c). This shows that extracellular CA enhances photosynthesis in MaB-floc SBRs treating paper mill UASB effluent (Fig. 5.1.e). In this way, CA might have indirectly increased the calcium removal by increasing the photosynthesis driven pH increase that facilitates calcite precipitation (Fig. 5.1.a; Fig. 5.1.c; Fig. 5.1.f). This is in line with the results of this study in batch reactors at initial neutral pH (Fig. 5.3.). The inhibition of extracellular CA might have also decreased the calcium removal by the lowered MaB-floc productivity in $SBR_{+CAinhib}$ compared to SBR (Table 5.2.). A slower metabolism can decrease the amount of negatively charged sites on photosynthetic microorganisms such as carboxylic groups (Obst et al., 2009) (Fig. 5.1.g). Similarly,

Table 5.2. *Performance of MaB-floc sequencing batch reactors treating paper mill UASB effluent without (SBR) and with addition of inhibitor of extracellular CA (SBR$_{+CAinhib}$)*

Parameter	**Concentration (mg L^{-1})**			**Removal efficiency (%)**		**Removal rate (mg L$_{reactor}$ $^{-1}$ day^{-1})**	
	Influent	**Effluent SBR**	**Effluent SBR$_{+CAinhib}$**	**SBR**	**SBR$_{+CAinhib}$**	**SBR**	**SBR$_{+CAinhib}$**
pH	7.36 ± 0.15^{b}	8.04 ± 0.14^{a}	7.55 ± 0.19^{b}	n.a.[1]	n.a.	n.a.	n.a.
TCa	364 ± 8^{a}	265 ± 39^{c}	312 ± 20^{b}	27.2 ±10.8^{a}	14.0 ± 5.4^{b}	24.7 ± 9.9^{a}	12.8 ± 4.9^{b}
TIC	274 ± 25^{a}	201 ± 23^{c}	246 ± 16^{b}	26.9 ± 8.3^{a}	10.4 ± 5.8^{b}	18.6 ± 5.9^{a}	7.2 ± 4.0^{b}
TOC	356 ± 40^{a}	151 ± 75^{b}	143 ± 61^{b}	57.7 ± 21.1	60.8 ± 19.4	51.3 ± 18.8	54.0 ± 17.3
TC	630 ± 41^{a}	351 ± 63^{b}	381 ± 62^{b}	44.3 ± 10.0	39.6 ± 9.9	69.7 ± 15.7	62.3 ± 15.6
TN	30.8 ± 2.5^{a}	7.2 ± 1.6^{b}	27.3 ± 2.4^{a}	76.5 ± 5.2^{a}	11.6 ± 7.9^{b}	5.9 ± 0.4^{a}	0.9 ± 0.6^{b}
TP	7.5 ± 0.5^{a}	2.0 ± 0.2^{c}	2.9 ± 0.5^{b}	73.4 ± 2.4^{a}	61.6 ± 7.0^{b}	1.4 ± 0.0^{a}	1.2 ± 0.1^{b}
Molar ratios							
TCa:TIC	0.40 ± 0.01	0.41 ± 0.08	0.38 ± 0.04	n.a.	n.a.	0.42 ± 0.20	0.56 ± 0.31
TC:TN	24 ± 2^{c}	57 ± 10^{a}	16 ± 3^{b}	n.a.	n.a.	14 ± 3^{b}	81 ± 20^{a}
TN:TP	9.1 ± 0.6^{b}	8.1 ± 0.7^{b}	22 ± 2^{a}	n.a.	n.a.	9.5 ± 0.3^{a}	1.7 ± 0.2^{b}

All values are averages from day 13 till day 48 of SBR run (16 sampling days for pH, TCa, TIC, TOC; 6 sampling days for TN and TP).

$^{a, b}$ Superscripts indicate significant differences between concentrations, removal efficiencies or removal rates according to one-way ANOVA and Tukey's post-hoc test or according to a Kruskal Wallis test with Bonferroni's correction ($p < 0.05$) when no homogeneity of variances or normal distribution of data was observed; [1] Not applicable

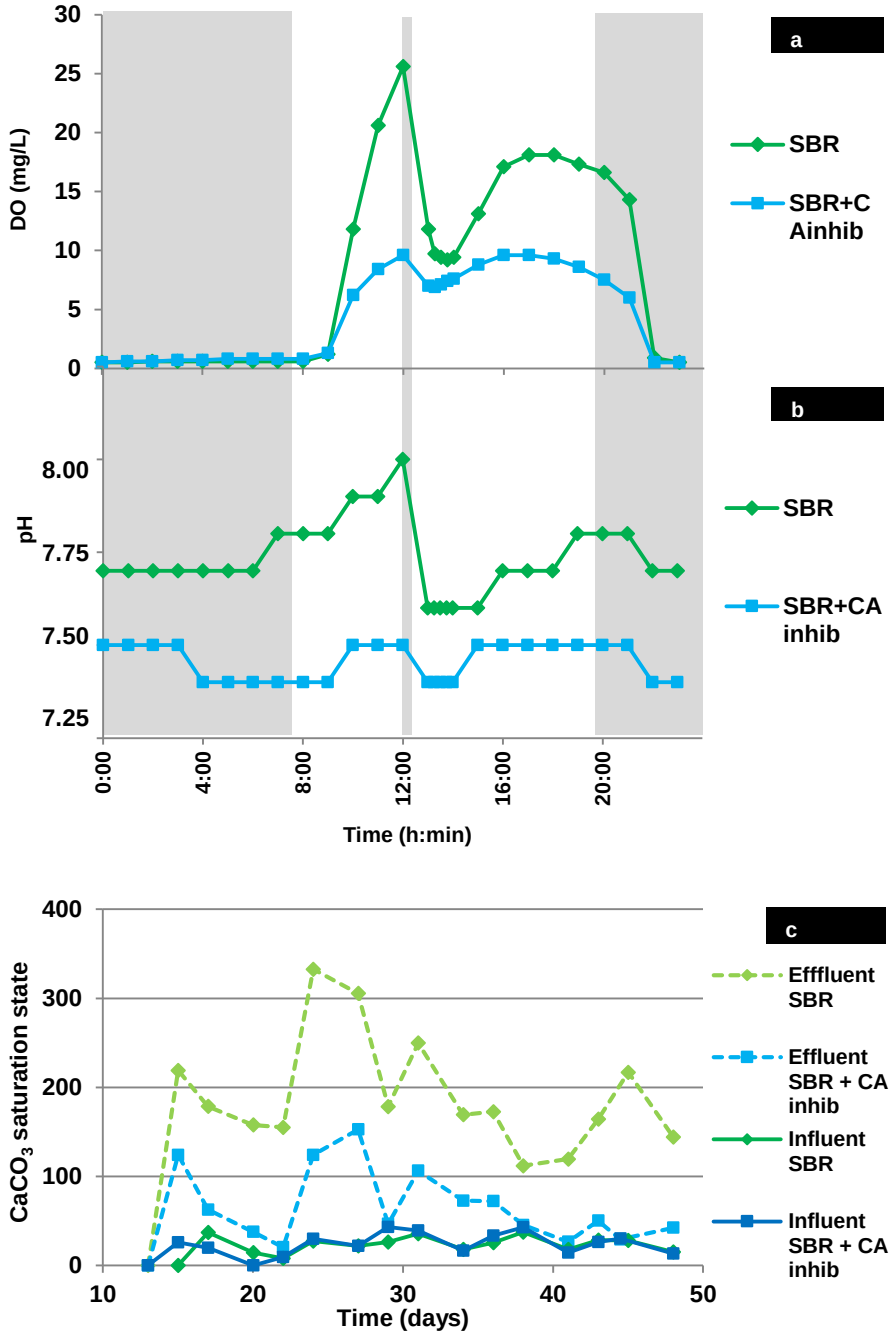
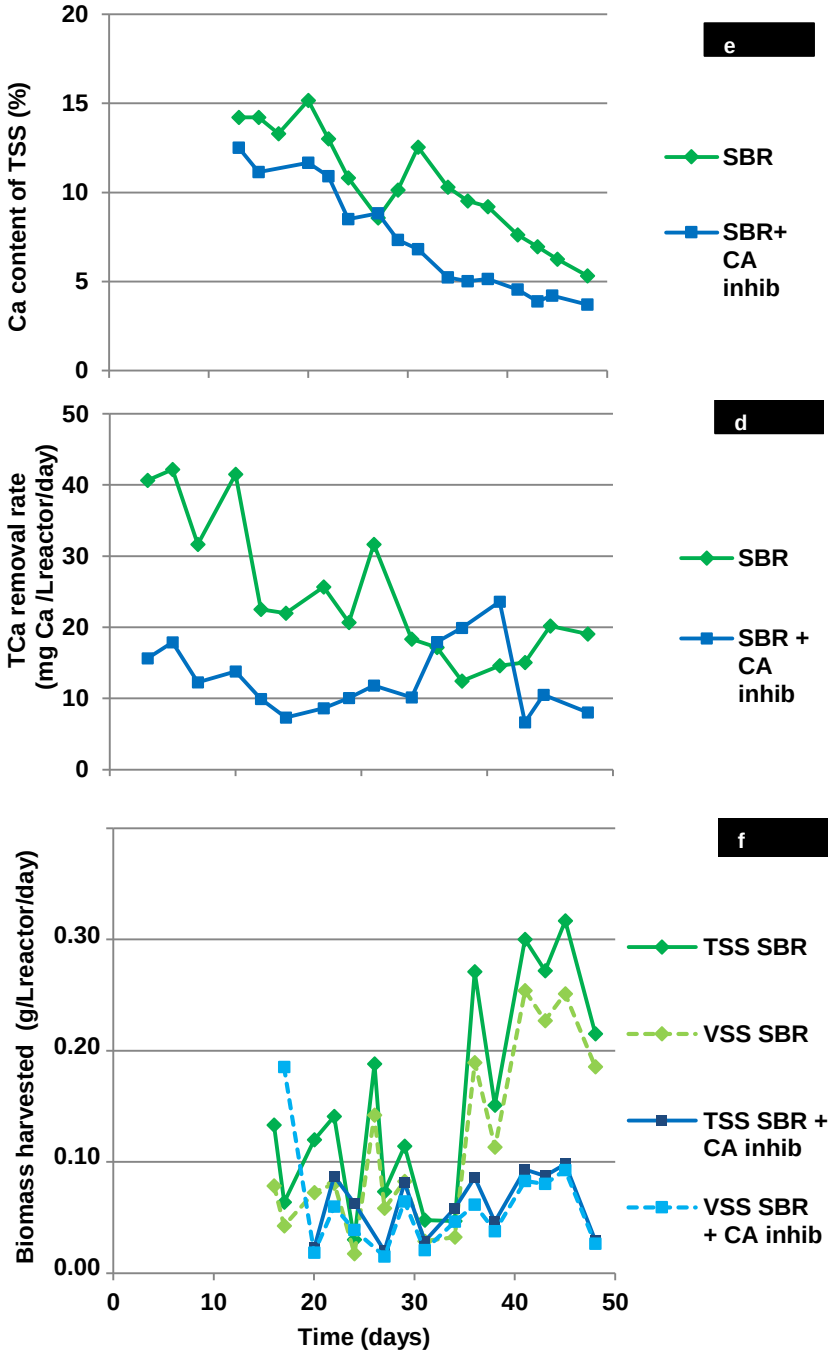


Fig. 5.4. *Effect of inhibiting extracellular CA on the treatment of paper mill UASB effluent in MaB-floc SBRs: reactor DO and reactor pH during SBR cycle 31 (a, b), $CaCO_3$ saturation state Ω_{CaCO3} (c), calcium removal rate (d), MaB-floc calcium content (e) and MaB-floc biomass harvesting (f)*

(d – f : on next page)



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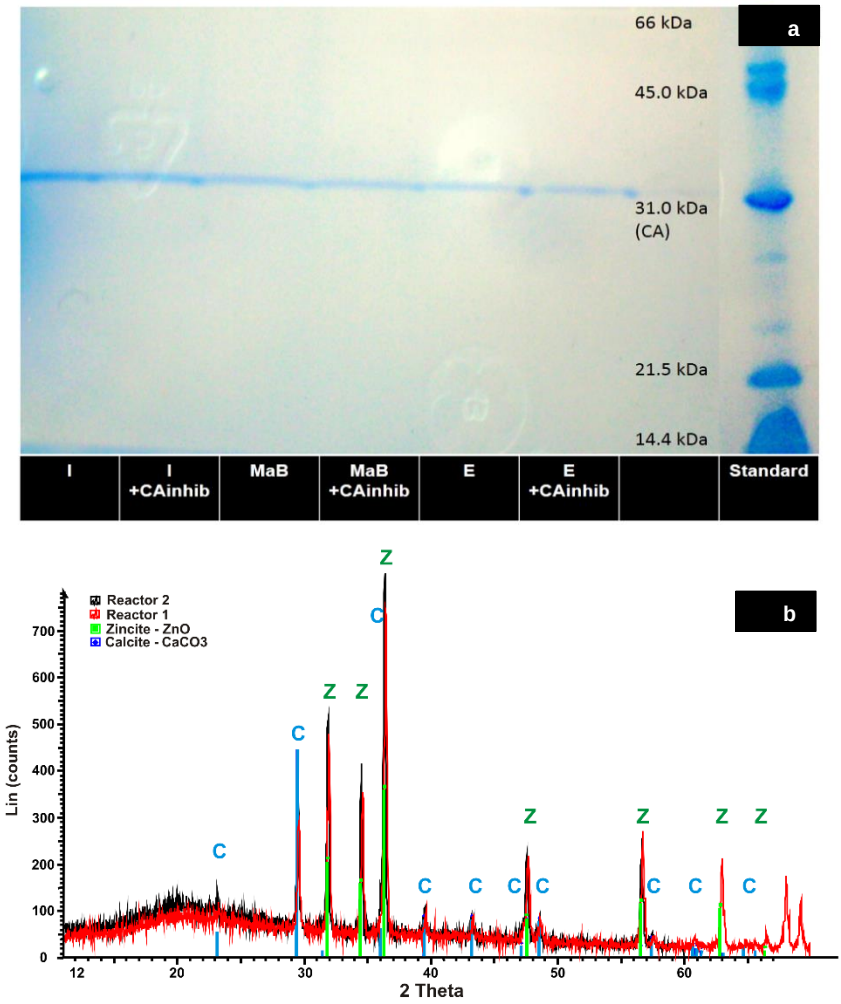


Fig. 5.5. *SDS-PAGE showing the presence of carbonic anhydrase in influent (I, I+CAinhib), MaB-flocs (MaB, MaB+CAinhib) and effluent (E, E+CAinhib) from SBR and* $SBR_{+CAinhib}$ *(a), and XRD analysis showing the presence of crystalline calcite in MaB-flocs of SBR (Reactor 1) and* $SBR_{+CAinhib}$ *(Reactor 2) (b)*

Ramanan et al. (2010) observed a slower growth of *Spirulina platensis* and *Chlorella* sp. by the addition of 1 mM AA, but the algae never ceased to grow.

The aforementioned results suggest that extracellular CA was present in the MaB-flocs SBRs. SDS-PAGE shows that a compound with a similar molecular mass as the control bovine CA (31.0 kDa) was present (Fig. 5.5.a). However, it could not be confirmed that this compound of 31 kDa was produced by MaB-flocs, since this compound was not only found in the MaB-flocs and in the effluent, but also in reactor

influent being paper mill UASB effluent (Fig. 5.5.a). The latter could have been produced during UASB treatment by several anaerobic microorganisms such as the methanogenic *Methanosarcina sp*. (Ferry et al., 2013). Further analyses by acetate cellulose electrophorese, is needed to confirm that CA was present, to distinguish the active CA isozymes originating from anaerobic and photosynthetic microorganisms, and to assess their role in calcium removal.

The TCa removal rate in SBR decreased in time. From day 36 till the end this TCa removal rate stabilized to values similar to $SBR_{+CAinhib}$ (Fig. 5.4.d). The SBR effluent pH remained stable and stayed higher than in $SBR_{+CAinhib}$ (Table 5.2.), the TIC removal rate of SBR remained stable (Table 5.2.), and the Ω_{CaCO3} of SBR remained above 100 (Fig. 5.4.e). This entails that the 'alkalinity engine', i.e. microbial metabolism and environmental conditions impacting the Ω_{CaCO3} (Dupraz et al., 2009), was not the only factor driving calcium removal in a MaB-floc SBR. Next to the photosynthetic increased pH (Fig. 5.1.a) and extracellular CA (Fig. 5.1.d), other factors were influencing calcium removal from paper mill UASB effluent in MaB-floc SBRs.

The MaB-floc architecture changed in time. During reactor operation, MaB-flocs in SBR evolved from a smaller filamentous floc to a larger, more open and very filamentous floc (Fig. 5.6.a; Fig. 5.6.b), while the calcium content of the MaB-flocs decreased (Fig. 5.4.e). The filamentous microorganisms that dominated the floc of both SBR and $SBR_{+CAinhib}$ was most probably the cyanobacteria *Phormidium* sp. (microscopic observations; Fig. 5.6.). These species have been observed in stromatolites (Jansson and Northen, 2010; Martinez et al., 2008) and waterwrats containing crystalline calcite (Garcia-Pichel and Wade, 2002). Also in MaB-flocs, crystals were observed (Fig. 5.6.). XRD diffractograms of MaB-flocs harvested at the end of the reactor operation (Fig. 5.5.b) confirmed that crystalline calcite was present in the harvested MaB-flocs. The presence of a bump between 10° and 30° of the XRD indicates the presence of amorph material. This amorph material may contain harvested MaB-flocs and/or amorphous calcium carbonate which can act as precursor of crystalline calcite (Dupraz et al., 2009). Quantitative calculations based on XRD indicated the presence of 5-10 % w/w crystalline calcite in both MaB-floc SBRs at the end of the reactor operation. At that day, MaB-flocs contained 5.2 % w/w calcium

in SBR and 3.7 % w/w in $SBR_{+CAinhib}$. This indicates that a major part of the calcium in MaB-flocs was present as calcite crystals.

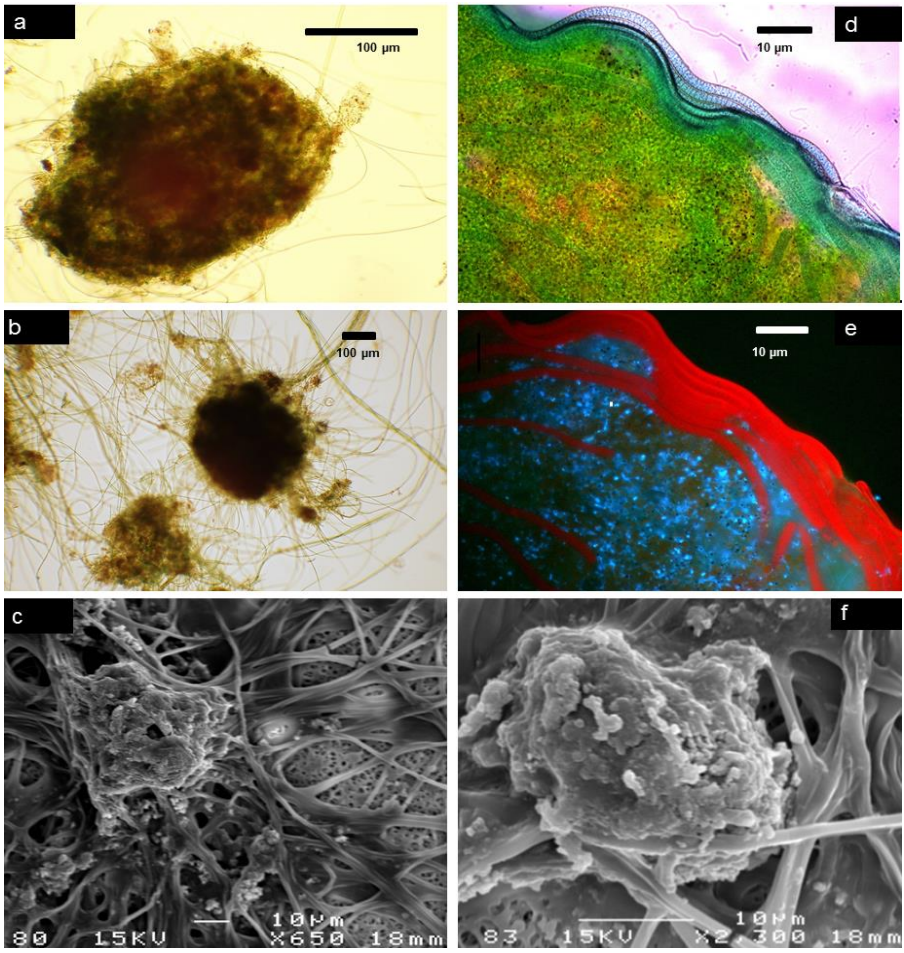


Fig. 5.6. *Micrographs of MaB-flocs treating paper mill UASB effluent: light microscopy showing the presence of crystals in MaB-flocs from SBR on day 13 (a) and on day 48 (b); light (d) and fluorescence microscopy (DAPI) (e) of the same MaB-floc from* $SBR_{+CAinhib}$ *showing the presence of blue light excitating calcite crystals; SEM microscopy showing a dumbbell shaped crystal next to the cell wall of Phormidium sp. of a MaB-floc from* $SBR_{+CAinhib}$ *(c, f)*

Interestingly, in all microscopically observed MaB-flocs, crystals were observed almost exclusively in the dense centre of the floc structure and not at the outer part. Therefore, loose, open MaB-flocs with widespread filaments contain fewer crystals as its floc centre represents only a small share of the floc. As the floc structure grew more open over time, a lower calcium content of MaB-flocs and a lower calcium

removal in time were noticed. Similar as in $CaCO_3$ precipitation of microbial mats, the enhanced calcite precipitation in closed MaB-flocs might be due to an increased cell death (Fig. 5.1.h), an increased EPS content (Fig. 5.1.i), a locally (at least temporary) increased pH and/or a locally increased I content (Dupraz et al., 2009).

The amount of MaB-flocs harvestings, necessary to maintain 1 g L^{-1} VSS, was less in the first period (day 0 till day 35) of reactor operation compared to the second period (day 36 till day 50) (Fig. 5.4.f). The average sludge retention time (SRT) in period 1 was 13 days and 6 days in period 2. Consequently, more aged MaB-flocs were present during this first period. A longer SRT might have resulted in more cell death in the MaB-flocs (Fig. 5.1.j). This might have led to a release of intracellular calcium (Fig. 5.1.m), and intracellular CA (Fig. 5.1.n), and/or breakdown of EPS releasing calcium (Fig. 5.1.k), increasing the likelihood of calcite formation. This phenomenon is in line with the fact that in stromatolite forming cyanobacteria, precipitation does not start until the EPS begins to break down which liberates Ca^{2+} bound to the polymers. Hereby are both calcium concentration and alkalinity increased (Martinez et al., 2008).

The above presented observations strongly suggest that biologically-influenced $CaCO_3$ mineralisation processes were involved in the calcium removal from paper mill UASB effluent. This was confirmed by the presence of dumbbell shaped crystals in MaB-flocs (Fig. 5.6.c; Fig. 5.6.f). These crystals can only be produced by biologically-influenced mineralization, a passive mineralization influenced by organic compounds (Dupraz et al., 2009). Compared to coccolithophoric algal species (Moheimani and Borowitzka, 2011), the maximum calcium content of MaB-flocs (Fig. 5.4.e) was similar, but the calcium removal rates were lower (11 % and 61.0 ± 17.3 mg Ca L^{-1} day^{-1} in *P. carterea*, and 12 % and 40.0 ± 1.9 mg Ca L^{-1} day^{-1} in *E. huxleyi*). The results of this study, however, indicate that further optimisation of calcium removal in MaB-floc SBRs is possible.

5.3.2.2. Removal of other wastewater parameters and effluent quality

In light of a MaB-floc SBR implementation for treatment of paper mill UASB effluent on industrial scale, the removal of common wastewater parameters, such as COD/TOC, TN and TP, is of large importance to reach the effluent discharge and/or reuse standards for these parameters.

TOC was significantly removed from paper mill UASB effluent in the MaB-floc SBR (Table 5.2.). This TOC removal efficiency was in line with a COD removal of 55-60 % during 12 days in batch reactors with a mixed culture of algal species (Tarlan et al., 2002), and of 60 % in ureolytic microbiological SBRs treating wastewater from the same origin as this study (Hammes et al., 2003). This TOC removal efficiency was low to similar compared to conventional aerated activated sludge reactors removing 57-97 % COD (Pokhrel and Viraraghavan, 2004). The common presence of recalcitrant COD in paper mill wastewater, such as lignin derivates (Bryant et al. 1988), and the DO oversaturation at the end of light phases the MaB-floc SBR (Fig. 5.4.a), suggest that an increase in HRT will not increase the TOC removal efficiency of the MaB-floc SBR.

TN and TP were significantly removed in the MaB-floc SBR (Table 5.2.). The European Best Available Technique Associated Emission Levels (BATAEL) for discharge of effluent in surface water are 3-7 mg TN L^{-1} and 0.3-0.7 mg TP L^{-1} (EC, 2013a). These were reached for TN, but not for TP. Compared to previous experiments with MaB-floc reactors, this TP removal was similar but the N removal was 3 to 7 times lower (Van Den Hende et al., 2011a, 2011b). In general the TN and TP concentrations in paper mill wastewater are generally so low that N and P addition is required to feed the microorganisms in a biological treatment process (EC, 2013a; Mobius, 2006). Therefore, a problem-solving strategy could be to decrease the nutrient addition and concomitantly decrease the wastewater treatment costs.

Inhibiting extracellular CA in $SBR_{+CAinhib}$ significantly decreased the effluent pH and the removal of TCa, TIC, TN and TP, but not of TOC (Table 5.2.). This demonstrates the key role of extracellular carbonic anhydrase in photosynthesis driven processes (Van Den Hende et al., 2012). Due to this decreased removal of TN and TP in $SBR_{+CAinhib,}$ the discharge norms for TN and TP were not reached (Table 5.2.) (EC, 2013a). The strongest effect of inhibiting extracellular CA was observed in the 6 times lower TN removal. This is reflected in the significantly different ratios of TC removal rate: TN removal rate and TN removal rate:TP removal rate in $SBR_{+CAinhib}$ compared to SBR (Table 5.2.). The stronger differences in removal rates of TN compared to TP might be explained by the decreased biomass productivity in $SBR_{+CAinhib}$ (Table 5.3.). Indeed, microalgae mainly remove TN by biomass growth, whereas TP can be removed via biomass growth, adsorption, precipitation and storage in phosphorous

bodies (Shilton et al., 2012). Overall, these results demonstrate the importance of extracellular CA in the treatment paper mill UASB effluent in MaB-floc SBRs.

The effluent pH increased during the light phase. However, by timing the effluent discharge shortly after the dark period, the effluent pH was kept below the current Flemish discharge norm of 9.5 (Vlarem II, 1995). Morever, the effluent pH was within the range of 5.9 to 8.5 needed for reuse in paper mills (Vito et al., 2010).

5.3.2.3. Dewatering of MaB-flocs

In this study, a very efficient way of harvesting of microalgal bacterial biomass was demonstrated on lab-scale, addressing one of the main bottlenecks of microalgal technology (Udom et al., 2013; Uduman et al., 2011; Grima et al., 2003). In general, microalgae are harvested by a first harvesting step that produces a microalgal slurry of 2-7 % TSS, for example by flocculant addition. This is followed by a second dewatering step that produces a microalgal paste of 15-25 % TSS, for example by centrifugation (Uduman et al., 2010). In this study, MaB-flocs were successfully harvested and dewatered in one single step by filter press with a relatively large pore size (200 µm; Fig. 5.7.) and a low pressure (40.2 kPa). No flocculants were added and the MaB-flocs did not need to settle prior to dewatering. This dewatering was stable and resulted in algal cakes of over 15 % dry matter in both SBR and $SBR_{+CAinhib}$ (Table 5.3.). A very high biomass recovery of over 99 %, calculated from the Chl*a* content in the press filtrate and MaB-flocs, was obtained (Table 5.3.). No significant effect of inhibiting extracellular CA on harvesting efficiency was observed (Table 5.3.).

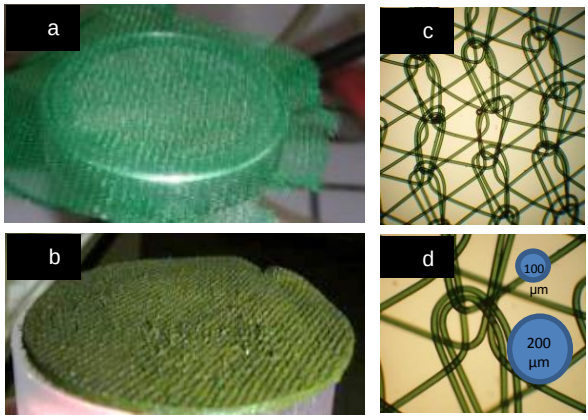


Fig. 5.7. *MaB-floc dewatering: pore size of the filtering screen (a, c, d), and dewatered MaB-flocs (b)*

The use of a filtering screen with a relatively large pore size is a huge advantage. Indeed, it increases the flow-through rate and lowers the operation costs. Besides, the use of non-settled MaB-flocs at a relatively low biomass concentration avoids blocking of the screen (Uduman et al., 2010). Moreover, based on LCA analyses, belt presses are a recommended algae dewatering technology if high solid contents are aimed at (Udom et al., 2013). This successful dewatering was due to bioflocculation and dominance of filamentous cyanobacteria in the MaB-flocs (Fig. 5.5.). Therefore it should be validated outdoors, since outdoor conditions might change the community structure and floc architecture of the MaB-flocs. Furthermore, the pressure, pressing time and filter pore size should be optimised on pilot scale.

Table 5.3. *MaB-floc productivity, calcium balance and harvesting by single-pass pressure sieving of MaB-flocs treating paper mill UASB effluent in sequencing batch reactors without (SBR) and with addition of CA inhibitor (SBR$_{+CAinhib}$)*

Parameter	Unit	SBR	SBR$_{+CAinhib}$
MaB-flocs[1]			
TSS just before harvesting	mg TSS L^{-1}	1906 ± 556^{a}	1437 ± 213^{b}
VSS just before harvesting	mg VSS L^{-1}	1353 ± 520	1116 ± 238
TSS just after harvesting	mg TSS L^{-1}	1533 ± 381	1254 ± 138
VSS just after harvesting	mg VSS L^{-1}	1196 ± 275	1089 ± 241
TSS productivity	mg TSS $L_{reactor}^{-1}$ day^{-1}	208 ± 140^{a}	70 ± 55^{b}
VSS productivity	mg VSS $L_{reactor}^{-1}$ day^{-1}	151 ± 112^{a}	59 ± 52^{b}
VSS:TSS	%	71.2 ± 9.8	77.5 ± 10.7
Calcium content of MaB-flocs	% of TSS	10.2 ± 3.0^{a}	7.6 ± 3.0
Calcium balance[1]			
Calcium removed by MaB-floc prod.	mg $L_{reactor}^{-1}$ day^{-1}	20.2 ± 11.3^{a}	3.9 ± 2.3$^{\mathbf{b}}$
Calcium discharged by effluent	mg $L_{reactor}^{-1}$ day^{-1}	65.8 ± 9.5^{b}	84.5 ± 3.0$^{\mathbf{a}}$
Difference[2]	mg $L_{reactor}^{-1}$ day^{-1}	4.4 ± 15.0	3.0 ± 4.0
Calcium loading by influent	mg $L_{reactor}^{-1}$ day^{-1}	90.5 ± 1.6	91.3 ± 2.4
Single-pass harvesting[3]			
Dry matter of MaB-flocs after sieving	%DM	16.9 ± 0.8	15.9 ± 1.0
Chlorophyll *a* content of filtrate	mg Chl*a* L^{-1}	0.05 ± 0.03	0.07 ± 0.11
Recovery of MaB-floc biomass	% of VSS	99.5 ± 0.4	98.1 ± 2.8

[1] All values are averages from day 13 till day 48 of SBR operation (15 sampling days); [2] Difference between the calcium removal plus discharge and the calcium loading; [3] All values are averages from day 13 till day 48 of SBR operation (9 sampling days); Superscripts indicate significant differences between 2 SBRs according to one-way ANOVA ($p < 0.05$) and Tukeys post-hoc test or according to Kruskal-Wallis test followed by a Mann-Whitney test in case no normality or homogeneity of variances was observed.

5.4. Conclusions

A novel and promising treatment for paper mill UASB effluent in a MaB-floc SBR is presented. Oxygen is provided via photosynthetic aeration and calcium is removed via bio-mineralization to calcite. Extracellular carbonic anhydrase enhanced the photosynthetic-driven calcium removal. MaB-flocs were efficiently harvested by a large-pore filter press, addressing one of the main bottlenecks of microalgal technology. These findings justify future research efforts towards optimal calcium removal, screening of more industrial wastewaters and outdoor feasibility of MaB-floc SBRs.

5.5. Acknowledgements

This work was supported by an assistant PhD grant from University College West-Flanders and Ghent University for S. Van Den Hende, and an Erasmus Scholarship for A. Rodrigues. The authors thank L. Desmet (Stora Enso) and C. De Winter (VPK) for their help with collecting wastewater and inoculum; A. Vergote (Howest) for advise on ICP analyses; A. Foubert (KULeuven) for discussion of XRD analyses; R. Swennen (KULeuven) for finding the first dumbbell crystal; H. Labeeuw (Howest) for installing the PLC; J. Van Wichelen (UGent) for algal species determination; D. De Buyser and K. Vercruysse (LEP-GEM) for help with CA analyses and use of equipment; Y. C. Arsan (UGent) for proof-reading the manuscript.

In het domein van schone technologie hebben we alle troeven in handen om Vlaanderen als wereldspeler op de kaart te zetten.

(Minister Lieten, 2013)

Picture on previous page:

Minister Ingrid Lieten holds a bottle with MaB-flocs in her office in Brussels and supports the INTERREG IVB NWE project EnAlgae.

Citation on previous page:

Minister Ingrid Lieten, 2013. Innovatief Vlaanderen Cleantech. ZO Magazine UNIZO 19, 42-43.

English translation: ‘With respect to clean-tech, Flanders has all assets which are needed to become a world player in this sector‘.

CHAPTER 6

Treatment of various industrial wastewaters by microalgal bacterial flocs in sequencing batch reactors

Abstract

Microalgal bacterial flocs in sequencing batch reactors (MaB-floc SBRs) represent a novel approach to wastewater treatment and biomass production by microalgae based on bioflocculation. To explore the potential of MaB-floc SBRs, wastewaters of various (agro-)industrial sectors were screened, specifically aquaculture, manure treatment, food-processing industry and chemical industry. These wastewater types resulted in significantly different removal rates of organic carbon, inorganic carbon, nitrogen and phosphorous. The low phosphorous concentrations in manure and aquaculture effluent (0.19-0.40 mg P L^{-1}) highlight the potential of MaB-floc SBRs for phosphorous polishing. The average biomass productivities of MaB-floc SBRs ranged from 0.14 to 0.26 g TSS $L_{reactor}^{-1}$ day^{-1}. Single-pass filter press harvesting recovered 79-99 % of MaB-flocs with 12-21 % dry matter. Although for some wastewaters further optimisation is needed to reach current discharge norms, our results demonstrate the wide applicability of MaB-floc SBRs.

Chapter redrafted after:

Van Den Hende, S., Carré, E., Cocaud, E., Beelen, V., Boon, N., Vervaeren, H. Treatment of various wastewaters by microalgal bacterial flocs in sequencing batch reactors. Submitted.

6.1. Introduction

During the past decade, there has been a true renaissance of interest in dual-purpose microalgal technology which couples wastewater treatment via photosynthetic aeration with the production of microalgal biomass (Olguín et al., 2012; Van der Ha et al., 2012; Su et al., 2011; Van Den Hende et al., 2011a; Muñoz and Guieysse, 2006; Gutzeit, 2006). This produced microalgal biomass can be an important bioresource for the production of valuable products, for example for biogas production (Zamalloa et al., 2012), feed ingredients (Natrah et al., 2013), and fine chemicals (Olguín et al., 2012; Van der Ha et al., 2012). Although potentially beneficial, however, these dual-purpose microalgae systems are rarely used on an industrial scale. A major hurdle is the high cost of microalgae harvesting (Udom et al., 2013; Uduman et al., 2010), which represents 25-60 % of the total microalgae production cost (Richmond, 2004; Molima Grima et al., 2003). Expensive conventional harvesting techniques such as chemical flocculation and centrifugation should be replaced by cheaper alternatives (Udom et al., 2013).

To address the high harvesting costs, the bioflocculation of microalgal biomass has been proposed as a promising option for wastewater treatment systems (Park et al., 2011b; Pittman et al., 2010). In contrast to non-flocculating microalgae systems, in microalgal floc systems the microalgae retention time can differ from the hydraulic retention time (HRT) of the wastewater; or -in algae culture terminology- the microalgal growth rate and culture dilution rate can be uncoupled (Van Den Hende et al., 2011a; Medina and Neis, 2007; Gutzeit, 2006). In this way, low-strength wastewaters can also be treated by high microalgal biomass densities.

To date, published research studies on the in situ bioflocculation of microalgae for wastewater treatment in (semi-)continuous reactors are still scarce. The reported research can be grouped into two different operation strategies. A first operation strategy is a continuous stirred reactor with settling tank and recirculation of the settled microalgal bacterial biomass (CSRSR), as have been reported for the treatment of sewage (Su et al., 2012b; Park et al. 2011a; Gutzeit, 2006) and paper mill wastewaters (Weinberger et al., 2012). A second operation strategy is a sequencing batch reactor with microalgal bacterial flocs (MaB-floc SBR). Studies on this are limited to the treatment of synthetic wastewaters (Chapter 2), sewage (Chapter 3) and paper mill effluent (Chapter 5). Compared to CSRSR, MaB-floc SBRs potentially

offer several advantages. First, there is no need for continuous pumping to recirculate the settled biomass from the settling tank to the reactor. Second, in MaB-floc SBRs floc settling occurs in the reactor at night. This decreases the high cost for the reactor stirring of open ponds (Richmond, 2004) and leads to long floc settling periods, which can enhance microalgae settling (Su et al., 2012a). Third, SBR operation is an effective way of obtaining fast settling microalgal flocs (Schwartz et al., 2010). Fourth, the sequenced influent addition and nutrient depletion can lead to nitrogen starvation, a well-known strategy for increasing the lipid content of microalgae (Van der Ha et al., 2013).

As an important first step towards their industrial implementation, this study further explores the potential of MaB-floc SBRs. The technical feasibility for the treatment of wastewaters of various (agro-)industrial sectors (aquaculture, manure treatment, food-processing industry and chemical industry) and simultaneous biomass production are assessed. Batch experiments are set up to develop MaB-inoculum and as a first screening of different wastewaters from each sector to select one wastewater per sector for treatment in MaB-floc SBRs. To assess the potential of MaB-floc SBRs for wastewater treatment, their nutrient (C, N, P) removal and effluent quality (pH, C, N, P) are comparatively evaluated. Moreover, the effect of the wastewater type on the MaB-floc properties and biomass productivities are examined. Aiming at lowering the microalgae harvesting costs, the potential for MaB-floc dewatering with a filter press with large pores (200 µm) is examined.

6.2. Materials and methods

6.2.1. Wastewaters

Several wastewaters from four (agro-)industrial sites were collected (Table 6.1). Aquaculture wastewaters ($A_{\_quar}$, $A_{\_small}$ and $A_{\_outgrow}$; Fig. 6.1.a) were drum filter effluents from pikeperch cultures of different fish growth stadia and stocking densities (Aquaculture Practice Centre of Inagro, Roeselare, Belgium). A_quar originated from quarantine pike perch cultures, $A_{\_small}$ from cultures of pikeperches smaller than 500 g and $A_{\_outgrow}$ from outgrowth trails with pikeperches larger than 500 g. Manure treatment wastewaters ($M_{\_pond1}$ and $M_{\_pond2}$; Fig. 6.1.b) were effluents collected from the first and second buffer pond (Innova Manure, Gistel, Belgium). Wastewater from the food-processing industry ($F_{\_UASB}$ and $F_{\_CAS}$; Fig. 6.1.c) was UASB effluent and

activated sludge effluent from a soy-processing company (Alpro, Wevelgem, Belgium). Wastewaters from chemical industry were mixtures of influent and effluent from an activated sludge reactor (BASF, Antwerp, Belgium) (Fig. 6.1.d). Influent: effluent mixtures (v: v %) of 100:0, 25:75 and 10:90 were used in the batch reactors and 50:50 was used in SBR ($C_{\_100I:0E}$, $C_{\_25I:75E}$, $C_{\_10I:90E}$ and $C_{\_50I:50E}$, respectively). Prior to feeding, all wastewaters were sieved through a 1-3 mm sieve to avoid tube clogging. All wastewaters were stored at 4 °C.

6.2.2. MaB-floc preculture in batch reactors

MaB-flocs were precultured in batch reactors in two steps. For each wastewater type, 800 mL wastewater, 300 mL of a consortium of microalgae/cyanobacteria collected on industrial sites (< 0.400 g volatilite suspended solids (VSS) L^{-1}) and 80 mL of MaB-flocs from previous cultures (0.090 g VSS L^{-1}) were mixed. For each wastewater type, two 500 mL Erlenmeyer flasks were filled with the algae/wastewater mixes and operated in batch mode with a 17 h light period with cycles of 0.5 h not stirring and 0.5 h stirring (210 rpm; Heidolph, UK), followed by a 7 h dark period without stirring. After 2.5 days, the reactor liquor was transferred to 5 L Erlenmeyers flasks and wastewater was added to obtain a working volume of 4 L. Over 6-7 days, a 14 h light:10 h dark cycle was applied with continuous stirring during the light phase and no stirring during the dark phase.

Fluorescent lamps (36/840, Philips, Belgium) provided a photosynthetic active photon flux density (PPFD) of around 100 µmol PAR photons m^{-2} s^{-1} at the water surface in the Erlenmeyer flask. Dissolved oxygen (DO), pH and temperature (T) were measured at the start and at the end of each light cycle. The $A664_b$:$A665_a$ ratio of MaB-flocs was determined daily. The diluted sludge volume index (dSVI) was determined at the end of each batch reactor.

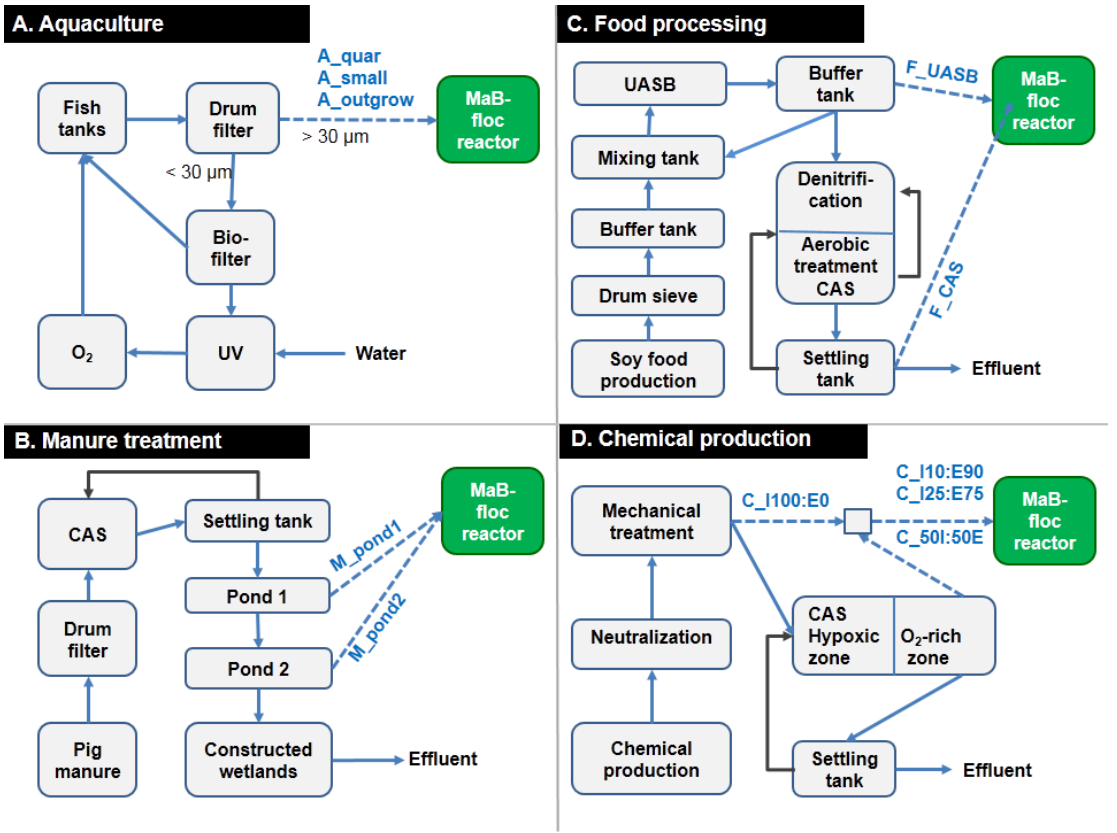


Fig. 6.1. *Flow charts of wastewater treatment systems of various (agro-)industrial sites showing origins of all wastewaters used in this study*

***Table 6.1**. Composition of wastewaters of various (agro-)industrial wastewaters used in batch experiments*

Para-meter	Unit	Aquaculture			Manure treatment		Food-processing industry		Chemical industry		
		A_quar	A_small	A_outgrow	M_pond1	M_pond2	F_UASB	F_CAS	C_100I:0E	C_25I:75E	C_10I:90E
pH		7.59	7.57	7.85	7.75	7.83	7.59	7.70	9.96	8.86	8.24
Turbidity	NTU	428	611	196	123	71	502	520	125	44	27
TCOD	mg O_2 L^{-1}	3110	1310	614	511	494	656	1163	1132	314	150
TOC	mg C L^{-1}	589	548	308	162	218	226	340	280	85	46
TIC	mg C L^{-1}	243	241	168	235	237	277	302	288	318	324
TC	mg C L^{-1}	823	789	476	396	455	503	642	568	403	370
TN	mg N L^{-1}	156	200	136	46.2	68.5	120	69.5	146	43.3	22.7
TP	mg P L^{-1}	166	75.6	16.2	0.41	0.50	14.5	18.1	5.4	3.0	2.5
Molar ratios											
TC:TN		6.17	4.59	4.09	10.0	7.75	4.89	10.8	7.12	10.7	12.7
TOC:TIC		2.39	2.27	1.83	0.672	0.920	0.816	1.13	0.972	0.267	0.142
TN:TP		6.17	5.86	18.5	249	305	18.3	8.49	38.1	32.7	30.3

6.2.3. Sequencing batch reactors

Wastewaters $A_{\_small}$, $M_{\_pond1}$, $F_{\_UASB}$ and $C_{\_50I:50E}$ were treated in SBRs, referred to as $SBR_{aquaculture}$, SBR_{manure}, SBR_{food} and $SBR_{chemical}$ respectively. SBRs were inoculated with 4 L reactor liquor of the batch reactors. SBRs were photobioreactors of 5 L with a working volume of 4 L as previous described (Van Den Hende et al., 2011a). The DO (Hanna Instruments, Belgium), pH and T (Jumo, Belgium) were logged every 30 s (only in $SBR_{aquaculture}$ and SBR_{manure}). Illumination by one halogen lamp (500 W, Silon CE-82-Y, Hong-Kong) for each SBR provided an average PPFD at the inner reactor wall (in µmol PAR photons m^{-2} s^{-1}) of 152 in $SBR_{aquaculture}$ and SBR_{manure}, 171 in SBR_{food} and 174 in $SBR_{chemical}$. Average reactor temperature in the SBRs was 31.2±4.0 °C, this was above room temperature due to heating by lamps. Each SBR was equipped by a diaphragm pump for influent feeding (Blackstone, USA), a peristaltic pump for effluent withdrawal (Watson Marlow, USA) and an overhead stirrer (210 rpm; Heidolph RZR 2020, Germany). Influents were stored at 4 °C and pumped into each SBR while being magnetically stirred (150-200 rpm; Heidolph, UK).

Two operation SBR modes were applied. The first consisted of a 7.75 h stirring phase in the light, 12 h settling phase in the dark, 3 h stirring phase in the light, 0.5 h settling phase in the dark, 0.25 h effluent withdrawal in the dark and 0.5 h influent feeding in the light while reactor stirring was carried out. The 3 h light phase before effluent withdrawal and influent feeding was introduced to aim for aerobic conditions while influent feeding. However, this resulted in gas bubble containing floating MaB-flocs, especially in $SBR_{aquaculture}$. Therefore, after the first 14 days for $SBR_{aquaculture}$ and SBR_{manure}, and after 10 days for SBR_{food} and $SBR_{chemical}$, a second SBR modus was applied consisting of a 11.5 h stirring phase in the light, a 8.75 h phase in the dark with 0.25 h stirring every 1-1.25 h, a 3 h settling phase in the dark, 0.25 h effluent withdrawal in the dark, and 0.5 h influent feeding in the light while reactor stirring was carried out. During the first 7 days in SBR_{food}, the HRT was 4 days to adapt to high COD concentrations, and was thereafter changed at 2 days. In all other SBRs, the HRT remained 2 days. All SBRs were operated for 35-36 days. No additional aeration of the reactors was performed. MaB-flocs were sampled three times a week and were analysed for total suspended solids (TSS), VSS, $A664_b$:$A665_a$, autotrophic index (AI) and chlorophyll *a* content (Chl*a*). MaB-flocs were frequently

harvested to maintain 1 g TSS L^{-1}, and were used for dSVI determination and filter press dewatering. Effluent and influent were sampled three times a week and analysed for pH, turbidity, total organic carbon (TOC), total inorganic carbon (TIC), total carbon (TC), total nitrogen (TN), total phosphorous (TP), total chemical oxygen demand (TCOD), soluble COD (SCOD) and biological oxygen demand (BOD_5).

6.2.4. Analytical protocols

The TSS, VSS, Chl*a*, $A664_b$:$A665_a$, AI and dSVI of MaB-flocs, and the TIC, TOC, TC, TN and pH of wastewaters were analysed as presented in Chapter 3. Microscopy and filter press dewatering were performed as presented in Chapter 5. Turbidity was measured with a turbidity meter (Hanna Instruments HI93703, Germany). TCOD was determined spectrophotometrically (Hach Lange DR2800, Belgium) (adjusted from Vito, 2002). The SCOD was determined on filtered wastewater (0.20 µm pore size syringe filter; Chromafil RC-20125, Germany). BOD_5 was determined following APHA et al. (2005). In the batch reactors, DO was measured with a DO meter (VWR DO 200, Belgium). PPFD was measured with a Li-250A light probe (Li-Cor, USA). Removal efficiencies and rates were calculated from effluent values measured three times per week compared to average influent values.

6.2.5. Statistics

Statistical analyses were performed using PASW Statistics 17.0 software (SPSS Incorporated, USA). Normal distribution of data was screened using the Shapiro-Wilk test and homogeneity of variances with Levene's test. In case of normal data distribution and homogeneity of variances, the differences in means were statistically analysed via one-way ANOVA and Tukey's post-hoc test ($p < 0.05$). Otherwise, Kruskal-Wallis followed by the Mann-Whitney post-hoc test including Bonferroni correction was used ($p < 0.05$). All means are given with standard deviations.

6.3. Results and discussion

6.3.1. Selection of wastewater and development of MaB-flocs

To select one wastewater from each (agro-)industrial site, batch reactors were set up and inoculated with a consortium of microalgae and cyanobacteria (hereafter

referred to as microalgae, to ease reading). In all batch reactors, the average $A664_b$:$A665_a$ ratio of day 4 till day 7 was above 1.62. This ratio is an indicator for the physiological condition of the microalgae (Van Den Hende et al., 2011a). A ratio of 1.7 indicates pure Chl*a* and a good physiological condition of microalgae; a ratio of 1.0 indicates pure pheophytin*a* (i.e. Chl*a* lacking a central Mg^{2+} ion) and no living microalgae. The results suggest that the consortium of microalgae employed did not exhibit severe toxicity in the tested wastewaters. In contrast to previous work on microalgal bacterial flocs (Su et al., 2012a; Weinberger et al., 2012; Van Den Hende et al., 2011a, 2011b; Gutzeit, 2006), bioflocculation of microalgae and bacteria was obtained for all screened wastewaters without the addition of activated sludge. The dSVI values ranged from 53 to 1274 mL g^{-1} TSS (Fig. 6.2.c).

The type of wastewater strongly affected the pH, DO and floc settling of MaB-flocs in batch reactors (Fig. 6.2.). In aquaculture wastewater $A_{\_outgrow}$, a steep pH increase (Fig. 6.2.a) and DO oversaturation (Fig. 6.2.b) were observed, indicating photosynthetic activity and aeration. Poor settling flocs with an extremly high dSVI (Fig. 6.2.c) were obtained with $A_{\_outgrow}$, due to the dominance of filamentous cyanobacteria in the MaB-flocs (Fig. 6.3.). In $A_{\_quar}$, increased and adequate MaB-floc settling was obtained. This was demonstrated by the low dSVI (Fig. 6.2.c). However, these flocs contained fewer microalgae (microscopic observation) and 50 % less Chl*a* (4.9 mg Chl*a* g^{-1} VSS in $A_{\_quar}$ compared to 7.8 in $A_{\_outgrow}$ at day 7). This was also reflected in the smaller increase of DO and pH. Wastewater of $A_{\_small}$ led to to values for dSVI, pH and DO ranging between those of $A_{\_outgrow}$ and $A_{\_small}$, and balanced presence of filamentous and non-filamentous microalgae (7.9 mg Chl*a* g^{-1} VSS at day 7) (Fig. 6.3.). Therefore, $A_{\_small}$ was selected as aquaculture wastewater for further treatment in SBR. The differences in floc characteristics, pH and DO might be due to the different TOC:TIC ratios of the wastewater (Table 6.1), as reported previously (Van Den Hende et al., 2011a). High TOC:TIC ratios result in bacteria-dominated MaB-flocs, while low TOC:TIC values result in cyanobacteria-dominated MaB-flocs. It is of high importance to balance both TOC and TIC to create a MaB-floc in a favourable operational window regarding both photosynthetic activity and settling properties.

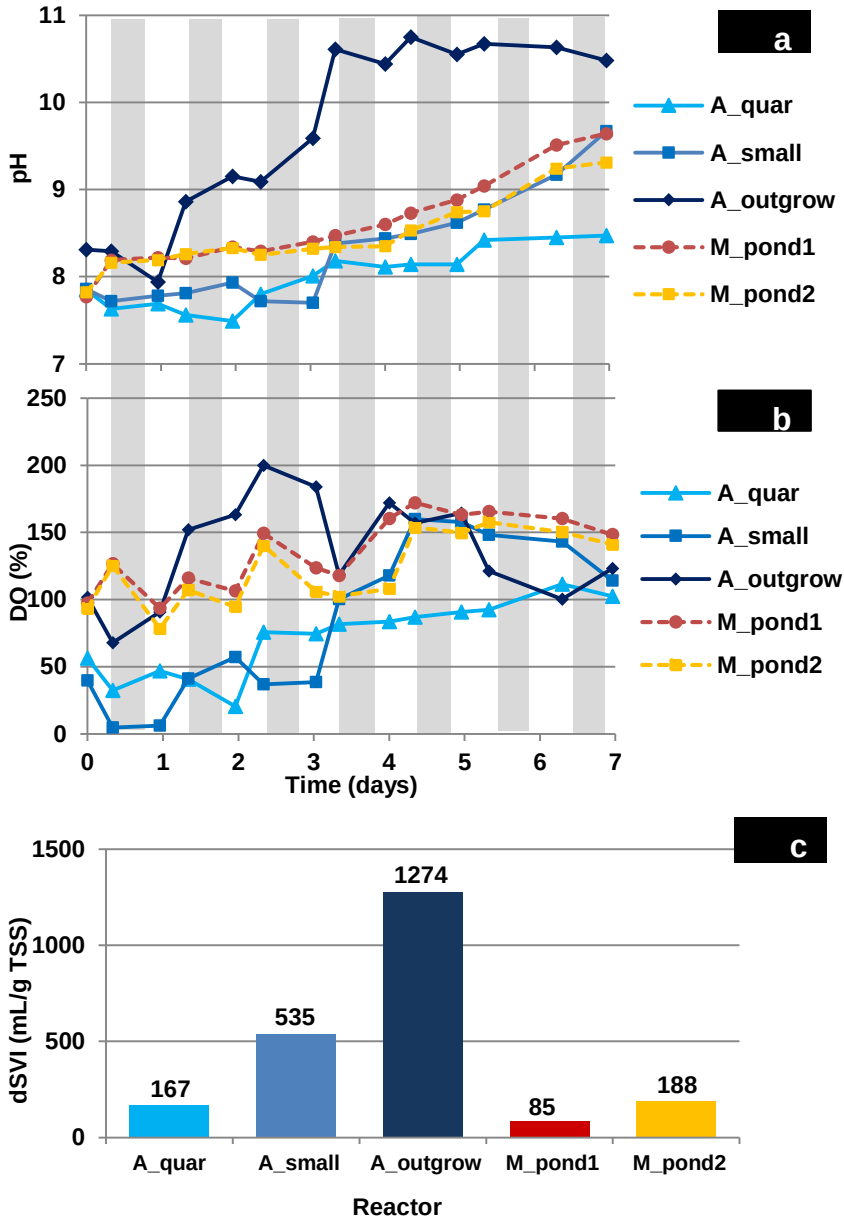
No large differences were obtained in the performances of batch reactors fed with different manure treatment effluents. In these batch reactors, the increase of pH

and DO (Fig. 6.2.a; Fig. 6.2.b) and increase of Chl*a* content of the reactor liquor were similar, indicating a similar photosynthetic activity. In both reactors, a more balanced amount of coccal microalgae and filamentous cyanobacteria were present in the MaB-flocs, leading to low dSVI values (Fig. 6.2.c). Wastewater of $M_{\_pond1}$ was selected for treatment in SBR because of its lower number of wastewater pretreatment steps.

In both reactors fed with different food-processing industry wastewaters, a similar pH increasing trend was observed (Fig. 6.2.d) and the DO increased to saturated conditions after 4-5 days, indicating photosynthetic activity in both reactors. In $F_{\_CAS}$, already after 3 days, the pH reached a value above the discharge norm of 9 (Fig. 6.2.d), due to the relatively high initial pH of this wastewater (Table 6.2.). In F_UASB, the pH remained below 9. The dSVI in $F_{\_UASB}$ was lower compared to $F_{\_CAS}$, demonstrating a better floc settling (Fig. 6.2.f). In $F_{\_UASB}$, DO started to increase after 2 days and reached DO saturation after 5 days (Fig. 6.2.d; Fig. 6.2.e). This demonstrated that photosynthetic aeration is possible in this TCOD-rich wastewater (Table 6.1.). Therefore, $F_{\_UASB}$ was selected as food-processing industry wastewater for further treatment in SBR. From an economic point of view, it could be more interesting to use photosynthetic aerated MaB-floc SBRs as alternative for mechanical aerated CAS compared to as tertiary treatment of CAS effluent. Moreover, microalgal bacterial treatment of methane saturated wastewaters, such as UASB effluent, has been shown to lower the emission of the greenhouse gas methane from the wastewater while converting it to biomass (Van der Ha et al., 2012).

Several mixes of CAS influent and effluent of the chemical industry were screened in batch reactors, all demonstrating an increase in DO (Fig. 6.2.e) and thus photosynthetic activity. All these batch reactors showed an adequate floc settling (Fig. 6.2.f). Increasing the percentage of effluent in these wastewater mixes decreased the TOC:TIC ratio (Table 6.1.). These increased values resulted in increased dSVI values after 6 days and in pH values above the discharge norm of 9 after 2-3 days in $C_{\_25I:75E}$ and $C_{\_10I:90}$ (Fig. 6.2.f, d). This again demonstrates the importance of the TOC:TIC ratio and reactor pH on floc settling, as reported previously (Van Den Hende et al., 2011b). In $C_{\_100I:0E}$, the pH remained below 9, but DO remained low until day 3 (Fig. 6.2.e) due to the high oxygen demand of this wastewater (Table 6.1.). Therefore, an intermediate mixture of 50 % influent: 50 % effluent was used in SBR.

Overall, the above results demonstrate that operating a consortium of microalgae in sequenced stirred batch reactors is an effective and simple method to efficiently obtain photosynthetic-active and adequate settling MaB-flocs in various industrial wastewaters.



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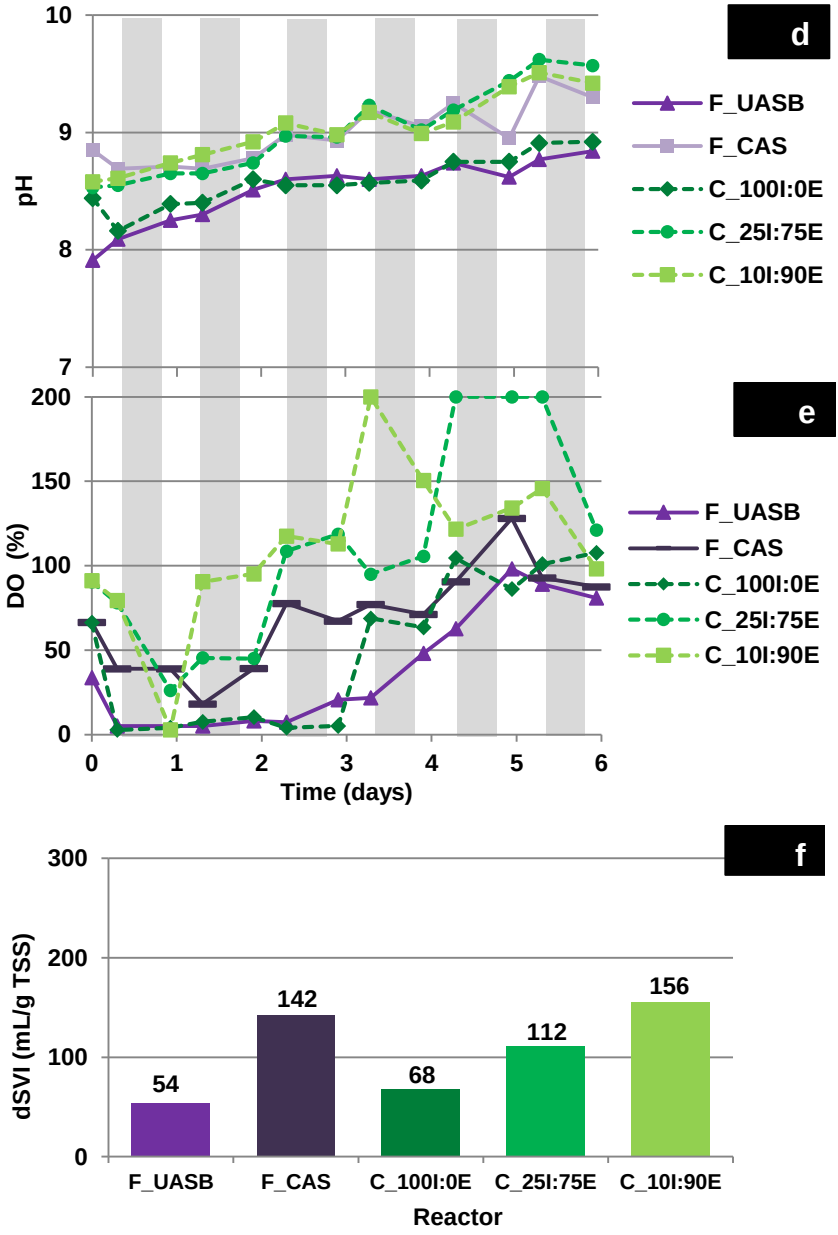


Fig. 6.2. *Reactor performance of microalgae bacteria batch reactors fed with wastewaters of various agro-industrial sites: pH (a,d), DO (b, e) and dSVI (c, f) (this and previous page)*

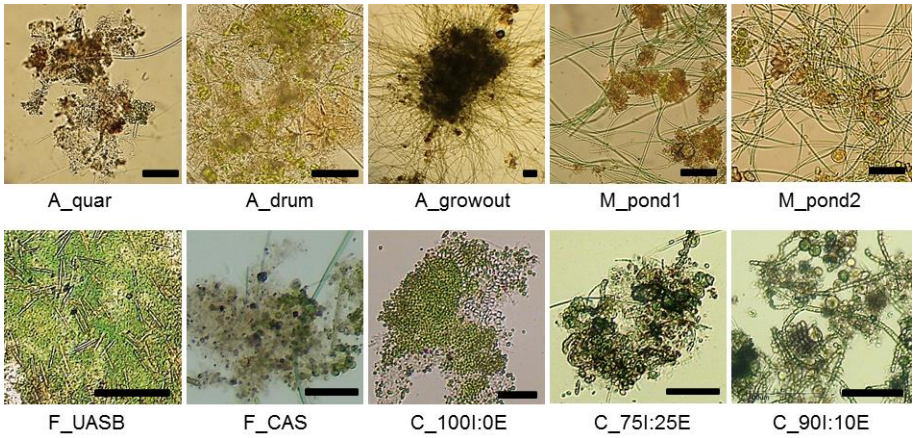


Fig. 6.3. *Micrographs of MaB-flocs from batch reactors grown on various wastewaters of aquaculture (A), manure treatment (M), food-processing industry (F), and chemical industry (C)*
Depicted scale bars measure 50 µm in length.

6.3.2. Wastewater treatment in SBR mode

Different wastewaters were treated in MaB-floc SBRs for 35-36 days with a HRT of 2 days. The effluent quality and nutrient removal in these MaB-floc SBRs were strongly dependent on the type of wastewater treated.

In $SBR_{aquaculture}$, the effluent pH and concentrations of TCOD, BOD, TN and TP were below the current discharge norms (Table 6.2.). A significant removal in turbidity, BOD_5, TCOD, TOC, TC, TN and TP was observed (Table 6.2.), resulting in removal efficiencies (in %) of 96 ± 1, 87 ± 11, 80 ± 7, 71 ± 21, 48 ± 14, 58 ± 11 and 89 ± 5, respectively (Fig. 6.4.). In this SBR, the average DO was 6.06 ± 6.66 mg L^{-1} and the average pH was 8.11 ± 0.72 (average of light and dark periods of day 16-35). Strong diurnal variations of DO and pH were observed in the MaB-floc SBR treating aquaculture wastewater (Fig. 6.5.; day 24-30 given as an example).

Next to TOC, a significant decrease in the TIC concentration of the wastewater was observed (Table 6.2.), resulting in a 36 ± 17 % TIC removal efficiency. For each 1.2 ± 0.9 mol of TOC removed from the influent, 1 mol of TIC was removed. This demonstrates that the in situ TIC production from TOC oxidation was lower than the TIC removal by photosynthetic growth, CO_2 emission and/or carbonate precipitation. Calcite precipitation has been demonstrated in MaB-flocs

treating paper mill wastewater (Chapter 5). Moreover, in MaB-flocs of $SBR_{aquaculture}$, crystals were observed (Fig. 6.6. in Section 6.3.4. Bioflocculation in MaB-floc SBRs). The wastewater turbidity decreased by a factor of 20 in $SBR_{aquaculture}$, This large removal of turbidity might be due to sequenced settling and/or dominance of *Phormidium* sp. in MaB-flocs (Fig. 6.6.). *Phormidium* sp. are cyanobacteria which can release turbidity removing bioflocculants (Bar-Or and Shilo, 1987). The low TP concentration in the effluent (Table 6.2.) highlight the large potential MaB-floc SBRs for phosphorous polishing and phosphorous recovery, which is of great interest nowadays (Shilton et al., 2012).

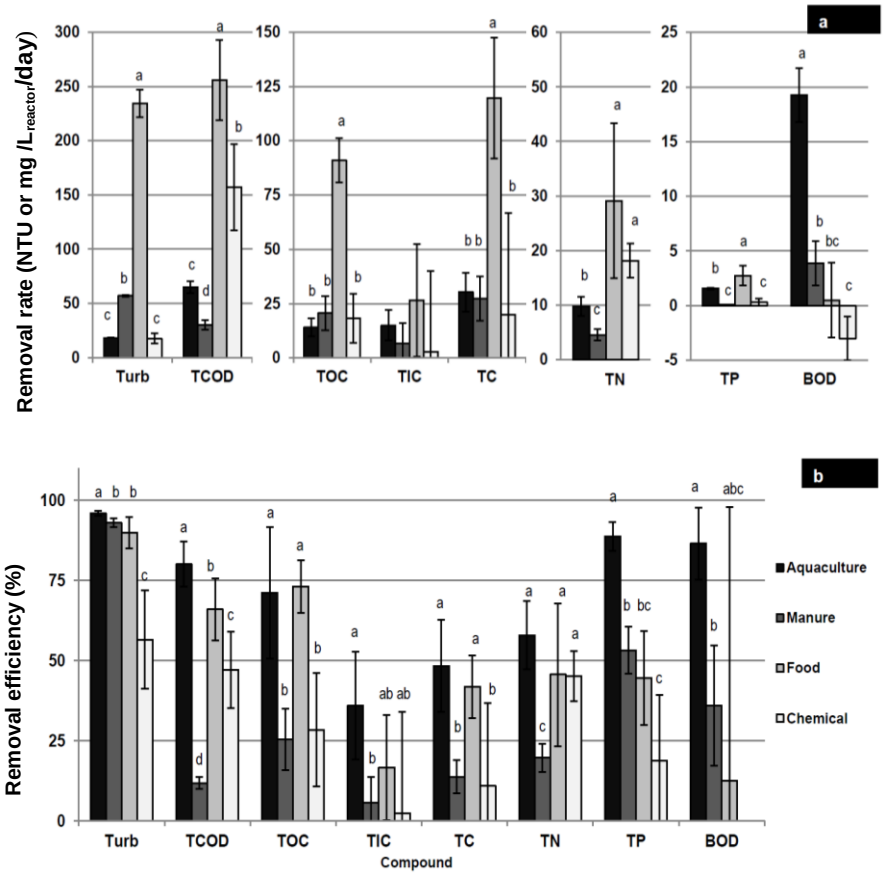


Fig. 6.4. *Removal rates (a) and removal efficiencies (b) of MaB-floc SBRs treating wastewater of aquaculture and manure treatment (averages from day 16 till 35), food-processing industry and chemical industry (averages from day 10 till 36)*

Table 6.2. *Composition of influent and effluent of MaB-floc SBRs treating wastewaters from various (agro-)industrial sectors*

Parameter	Unit	Aquaculture[1]			Manure treatment[1]		
		Influent	**Effluent**	**Norm[3]**	**Influent**	**Effluent**	**Norm**
pH		7.59 ± 0.28	7.81 ± 0.24	6.0-9.5	7.75 ± 0.29	7.64 ± 0.28	6.0-9.5
Turbid	NTU	38.0 ± 4.2*[4]	1.5 ± 0.3		123 ± 48*	8.6 ± 1.6	
TCOD	mg L^{-1}	162 ± 39*	32 ± 11	125	511 ± 26*	450 ± 9	125
BOD_5	mg L^{-1}	45 ± 12*	6 ± 5	25	22 ± 7	14 ± 4	25
TOC	mg L^{-1}	39 ± 10*	11 ± 8		162 ± 28*	120 ± 16	
TIC	mg L^{-1}	84 ± 10*	54 ± 14		235 ± 20	221 ± 19	
TC	mg L^{-1}	125 ± 11*	65 ± 18		396 ± 13*	342 ± 20	
TN	mg L^{-1}	33.7 ± 4.2*	14.2 ± 3.6	99.8[5]	46.2 ± 2.6*	37.1 ± 2.1	15
TP	mg L^{-1}	3.52 ± 0.59*	0.40 ± 0.16	24	0.41 ± 0.11*	0.19 ± 0.03	2
Parameter	**Unit**	**Food-processing industry[2]**			**Chemical industry[2]**		
		Influent	**Effluent**	**Norm**	**Influent**	**Effluent**	**Norm**
pH		7.56 ± 0.18*	8.34 ± 0.17	6.5-9.0	9.33 ± 0.37	9.18 ± 0.26	6.5-9.0
Turbid	NTU	521 ± 114*	52.6 ± 25.4		62.5 ± 28.0*	27.1 ± 9.6	
TCOD	mg L^{-1}	775 ± 172*	264 ± 74	120	666 ± 117*	352 ± 79	125
BOD_5	mg L^{-1}	8 ± 0	7 ± 7	25	0 ± 0	8 ± 0*	
TOC	mg L^{-1}	249 ± 62*	67 ± 20		128 ± 29*	92 ± 23	
TIC	mg L^{-1}	317 ± 43*	264 ± 52		235 ± 53	230 ± 74	
TC	mg L^{-1}	570 ± 94*	331 ± 56		364 ± 70	324 ± 94	
TN	mg L^{-1}	128 ± 14*	69.3 ± 28.4	15	80.2 ± 12.9*	44.0 ± 6.2	15
TP	mg L^{-1}	12.3 ± 1.8*	6.81 ± 1.80	2	3.31 ± 1.09	2.69 ± 0.68	2

[1] Averages of 7-9 sampling days from day 16 till day 35 of SBR operation, except for BOD_5 where only 4 sampling days; [2] Averages of 9-12 sampling days from day 10 till day 36 of SBR operation, except for BOD_5 where only 3 sampling days; [3] Current (01/10/2013) effluent discharge norms applied by the companies were the wastewaters were selected; [4] Significant ($p < 0.05$) differences between influent and effluent of the same wastewater type are indicated with an asterisk according to one-way ANOVA and Tukeys post-hoc test or Kruskal-Wallis test followed by a Mann-Whitney test; [5] Sum of TKN, $N\text{-}NO_3^-$, $N\text{-}NO_2^-$.

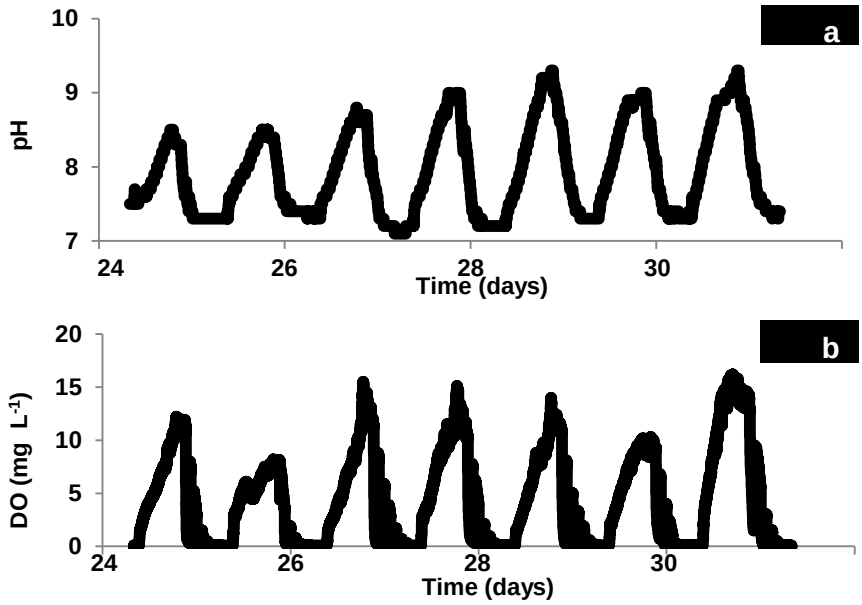


Fig. 6.5. *Diurnal fluctuations of pH (a) and DO (b) in the MaB-floc SBR treating aquaculture wastewater*

The effluent of SBR_{manure} reached the current discharge norms for pH, BOD_5 and TP, but not for TCOD and TN (Table 6.2.). A significant removal of turbidity, TCOD, TOC, TC, TN and TP was observed in SBR_{manure} (Table 6.2.). This resulted in removal efficiencies (in %) of 93 ± 1, 12 ± 2, 25 ± 10, 14 ± 5, 20 ± 4 and 53 ± 7, respectively (Fig. 6.4.b). In this reactor, no significant removal of BOD_5 from the influent was observed, but the BOD_5 concentration in the influent was relatively low (Table 6.2.). The average DO was 5.34 ± 3.22 mg L^{-1} and the average pH was 7.69±0.15 (average of light and dark periods of day 16-35) in SBR_{manure}. Both DO and pH increased in time in the light phase and decreased in the dark phase (data not shown). The BOD_5:TCOD ratio of the influent was 0.15 ± 0.10. This low ratio shows the presence of recalcitrant COD in this CAS effluent from manure treatment, and explains the low TCOD removal efficiency (Fig. 6.4.b). The molar TN:TP ratio of the influent and effluent, the molar ratio of the TN removal rate and TP removal rate (Table 6.3.), together with the low TP concentration of the effluent (Table 6.2.), suggest that phosphorous was limiting the nitrogen removal. The addition of dark-coloured CAS influent to light-coloured CAS effluent of the manure treating plant could enhance the TN:TP ratio of the influent, but will also lead to a darker wastewater

colour affecting autotrophic microalgal growth. Therefore, a balanced mixture for manure treatment wastewaters needs to be determined.

Table 6.3. *Molar TC:TN and TN:TP ratios of nutrient concentrations of the influent, the effluent and the removal rates of MaB-floc SBRs treating various (agro-)industrial wastewaters*

SBR	Type	TC:TN	TN:TP
Aquaculture	Influent	4.6 ± 0.7	20.6 ± 4.0
	Effluent	5.3 ± 1.9	121 ± 95
	Removal rate	4.1 ± 1.3	14 ± 3
Manure	Influent	10.0 ± 0.7	263 ± 57
	Effluent	10.7 ± 1.0	436 ± 74
	Removal rate	7.91 ± 4.41	92 ± 20
Food	Influent	4.55 ± 2.30	20.1 ± 8.6
	Effluent	7.38 ± 3.24	22.0 ± 5.8
	Removal rate	4.62 ± 1.49	22.9 ± 6.3
Chemical	Influent	5.26 ± 1.29	57.9 ± 17.8
	Effluent	9.31 ± 1.98	31.7 ± 16.1
	Removal rate	0.36 ± 2.58	142 ± 324

In SBR_{food}, effluent discharge norms were only met for pH and BOD_5, but not for TCOD, TN and TP. A significant removal of turbidity, TCOD, TOC, TIC, TC, TN and TP was observed (Table 6.2.), resulting in removal efficiencies (in %) of 90 ± 5, 66 ± 10, 73 ± 8, 17 ± 16, 42 ± 10, 56 ± 22 and 45 ± 15, respectively (Fig. 3b). Despite the fact that in this food-processing UASB wastewater, only 0.9 % of the TCOD was BOD_5, a short HRT of 2 days led to an average TCOD removal of 66 % in SBR_{food} (Fig. 6.3.b). Further research is needed to confirm whether this because UASB sludge particles were removed during settling in the SBR but not oxidised during BOD_5 determination, and/or photosynthesis induced metabolism occurred which resulted in some surplus TCOD that was vulnerable to biological degradation. Indeed, the enhancement of degradation of anaerobic bacteria by antibiotic excretion by cyanobacteria was previously observed (Caceido et al., 2010). Another possibility is that extensive removal of TCOD occurred via volatilisation of organic compounds, but since the influent of SBR_{food} contained only 47 mg SCOD L^{-1} (6 % of TCOD), this option is unlikely. The SCOD was for 100 % removed, accounting for a removal rate of 24 mg SCOD $L_{reactor}^{-1}$ day^{-1} (average of three sampling days). An HRT of 2 days

was applied to compare reactor performances for the different wastewaters. However, based on the results obtained in SBR_{food}, it seems worthwhile to investigate whether increasing the HRT for this nutrient-rich wastewater would result in meeting the discharge norms for the effluent.

The molar TC:TN ratios of the effluent and of the removal rates in SBR_{food} were similar and the molar TN:TP ratios for effluent and removal rates did not greatly differ (Table 6.3.). This suggests that a further balanced TC:TN:TP removal might be possible by increasing the HRT. However, this should be confirmed in further experiments, as recalcitrant C, N and P and change in nutrient affinities might change these molar ratios of removal rates. Despite the tenfold decrease in turbidity, the turbidity present in the effluent of SBR_{food} was still relatively high and fluctuating (Table 6.2.). This was due to the temporary presence of non-settling microalgae *Scenedesmus* sp. present in the reactor (microscopic observations). Based on the average TOC concentration in the effluent and the 49-51 % carbon content in *Scenedesmus* sp. (Tang et al., 2011), the average *Scenedesmus* sp. density in the effluent could have been at most 15 % of the VSS concentration of the reactor liquor.

Only in $SBR_{chemical}$ was the current discharge norm for pH not reached. This was due to the high pH of the influent (Table 6.2.). No current discharge norms were reached for TCOD, TN or TP (Table 6.2.). A significant removal of turbidity, TCOD, TOC and TN was observed, but not for TC and TP (Table 6.2.). Removal efficiencies (in %) of 57 ± 15, 47 ± 12, 29 ± 18, 2 ± 31, 11 ± 26, 45 ± 8 and 19 ± 21 were obtained for the turbidity, TCOD, TOC, TIC, TC, TN and TP, respectively. The influent of $SBR_{chemical}$ contained 379 mg SCOD L^{-1} (57 % of TCOD) which was for 64 % removed, accounting for 121 mg SCOD $L_{reactor}^{-1}$ day^{-1} (average of three sampling days). On the other hand, no BOD_5 was found in the wastewater (Table 6.2.). The strong smell of the off gas of $SBR_{chemical}$ indicates that at least some of this SCOD must have been volatilised during the reactor operation. The average molar ratio of the TC removal rate:TN removal rate was below one. This means that on average more TN was removed compared to TC (Table 6.3.). Further research is needed to confirm whether this is due to ammonia volatilization at a pH level of above 9 (Richmond, 2004) or denitrification during anoxic/anaerobic dark periods (Su et al., 2012a).

Significant differences were not only found in the removal efficiencies, but also in the removal rates for TCOD, TC, TN and TP among the SBRs treating different wastewaters (Fig. 6.4.). Overall, of all screened wastewaters, the aquaculture wastewater seemed to be the best candidate for wastewater treatment in a MaB-floc SBR. With an HRT of 2 days, all current discharge norms (pH, BOD_5, TCOD, TN, TP) and the overall highest removal efficiencies of all screened wastewater parameters were obtained with this wastewater (Fig. 6.4.). The molar ratios of TC removal rate:TN removal rate ranged from 0.36 to 7.91, while those of TN removal rate:TP removal rate ranged from 14 to 142. These ranges are much wider than reported for the TC:TN and TN:TP ratios of microalgal biomass (Geider and Le Roche, 2002). This demonstrates that microalgal biomass productivity is not the only mechanism for nutrient removal from wastewater in a MaB-floc SBR. Other N and P removal mechanisms which may have played a role are settling, adsorption of particulate matter and dissolved compounds, denitrification, volatilisation of wastewater compounds, luxury uptake and precipitation. Moreover, this means that the nutrient removal from wastewater by a microalgal consortium cannot be simply calculated based on the biomass productivity and Redfield TC:TN:TP ratio of microalgal biomass (106:16:1) (Geider and Le Roche, 2002). Overall, the removal rates for COD, TN and TP were equal or higher when compared to previous studies with microalgal reactors treating wastewater (Table 6.5. presented in Section 6.3.4. MaB-floc productivity in SBRs and dewatering). This demonstrates the large potential of MaB-floc SBRs for the microalgal treatment of wastewater.

6.3.3. Bioflocculation in MaB-floc SBRs

Adequate floc settling is crucial for MaB-floc SBRs, as it avoids washout of MaB-flocs via effluent withdrawal, thereby leaving a clear effluent. During the first 14 days of $SBR_{aquaculture}$ operation, flotation of MaB-flocs which contained gas bubbles was observed in the DO-saturated reactor liquor. During this period, effluent was withdrawn after a 3 h light period to aim for aerobic conditions while influent feeding. This problem was successfully addressed by adjusting the reactor operation. In this second SBR operation period, MaB-flocs were discontinuously stirred at night in order to remove gas bubbles from the MaB-flocs, and effluent was withdrawn at the end of the dark period. This led to adequate dSVI values in $SBR_{aquaculture}$ (Table 6.4.).

***Table 6.4**. Biomass characteristics, settling and dewatering of MaB-flocs from SBRs treating various (agro-)industrial wastewaters*

Parameter	Unit	SBR			
		Aquaculture	**Manure**	**Food**	**Chemical**
Biomass[1, 2]					
TSS before harvest	g TSS L^{-1}	1.830 ± 0.333^{a}	1.109 ± 0.343^{bc}	1.407 ± 0.410^{ab}	0.980 ± 0.331^{c}
VSS before harvest	g VSS L^{-1}	0.782 ± 0.127^{b}	0.544 ± 0.143^{b}	1.208 ± 0.295^{a}	0.612 ± 0.145^{b}
TSS after harvest	g TSS L^{-1}	1.263 ± 0.304^{a}	0.920 ± 0.288^{ab}	1.176 ± 0.258^{ab}	0.945 ± 0.265^{b}
VSS after harvest	g VSS L^{-1}	0.534 ± 0.090^{b}	0.452 ± 0.125^{b}	1.035 ± 0.222^{a}	0.577 ± 0.132^{b}
VSS:TSS	%	43.3 ± 6.1^{b}	50.0 ± 5.0^{b}	87.0 ± 7.8^{a}	64.9 ± 10.5^{b}
TSS productivity	g TSS $L_{reactor}^{-1}$ day^{-1}	0.236 ± 0.073	0.136 ± 0.100	0.257 ± 0.116	0.236 ± 0.294
VSS productivity	g VSS $L_{reactor}^{-1}$ day^{-1}	0.109 ± 0.030^{b}	0.068 ± 0.067^{c}	0.223 ± 0.090^{a}	0.122 ± 0.141^{bc}
Chl*a*	mg Chl*a* g^{-1} VSS	5.67 ± 0.81^{a}	2.63 ± 0.92^{b}	8.42 ± 2.56^{a}	8.83 ± 5.90^{a}
$A664_a$:$A665_b$	-	1.72 ± 0.04^{a}	1.70 ± 0.04^{a}	1.68 ± 0.06^{a}	1.52 ± 0.09^{b}
AI	(mg VSS mg^{-1} Chl*a*)	180 ± 28^{b}	417 ± 125^{a}	132 ± 48^{b}	152 ± 72^{b}
Floc settling[3]					
dSVI	mL g^{-1} TSS	193 – 272	91 - 150	69 - 81	41-142
Settled floc density	g TSS L^{-1}	5.2 – 3.7	6.7 – 11	12 - 15	7-24
Floc dewatering[2, 4]					
Dry matter flocs	% DM	21.1 ± 2.4^{a}	18.0 ± 2.2^{b}	11.8 ± 1.5^{c}	15.3 ± 1.9^{bc}
Biomass recovery	%	99.2 ± 0.7^{a}	96.6 ± 1.9^{b}	88.0 ± 5.6^{c}	79.0 ± 27.2^{abc}

[1] Averages of 8-12 sampling days; [2] Significant ($p < 0.05$) differences between wastewater types are indicated with a different subscript (a, b, c) according to one-way ANOVA and Tukeys post-hoc test or Kruskal-Wallis test followed by a Mann-Whitney test with Bonferroni correction; [3] Ranges of 2 sampling days for aquaculture/manure, and of 3 sampling days for food/chemical; [4] Averages of 9 sampling days for aquaculture/manure, of 5 sampling days for food, and of 3 sampling days for chemical industry wastewater

To compare all reactor performances, all SBR operations were adjusted in this way. In SBR_{food}, the dSVI was adequate and remained stable (Table 6.4.). In $SBR_{chemical}$, dSVI values improved from 142 mL g^{-1} TSS at day 15 to 41 mL g^{-1} TSS at day 24 and 58 mL g^{-1} TSS at day 36. Compared to the other SBRs, dSVI values of $SBR_{aquaculture}$ and SBR_{manure} were higher but still low enough to avoid MaB-floc loss via withdrawal of 50 % of the reactor volume. In all SBRs in this study, the densities of settled MaB-flocs were in the same range of those reported in previous studies (Van Den Hende et al., 2011a, 2011b).

The physiological condition of microalgae, evaluated by means of the $A664_b$:$A665_a$ ratio, was excellent in all SBRs, except for $SBR_{chemical}$ (Table 6.4.). In the latter SBR, the $A664_b$:$A665_a$ ratio remained around 1.5. This demonstrates the presence of pheophytin *a*, which is a breakdown product of Chl*a*. This suggest that moderate toxicity of the photosynthetic microorganisms to the wastewater of chemical industry could have been present, or that a lack of nutrients had this effect. However, these MaB-flocs never ceased to grow.

To assess the proportion of microalgae and bacteria in MaB-flocs, the AI, a standard method used in periphyton with values ranging from 50 to 350 (APHA et al., 2005), was determined. The AI values for MaB-flocs of $SBR_{aquaculture}$, SBR_{feed} and $SBR_{chemical}$ were low to moderate in all reactors (Table 6.4.). This indicates a dominance of photosynthetic microorganism in these MaB-floc communities. In contrast, the average AI of MaB-flocs of SBR_{manure} was significantly higher compared to those of the other SBRs and increased over time, indicating the increasing dominance of non-photosynthetic microorganisms, as confirmed by microscopic observations. The highest content of Chl*a* of MaB-floc VSS was obtained in SBR_{food} and the lowest was observed in SBR_{manure}, which was 2-8 times lower than previous results concerning sewage-fed MaB-flocs (Van Den Hende et al., 2011a). The Chl*a* content of the MaB-flocs of $SBR_{chemical}$ strongly decreased in time from 17.9±4.0 (day 10-16) to 7.7±0.8 (day 17-23) and stabilised at 4.8±0.7 mg Chl*a* g^{-1} VSS (day 24-36). This decrease might have been due to the moderate toxicity of this wastewater of the chemical industry to the microalgae.

Microscopic observations showed a good incorporation of photosynthetic microorganisms in MaB-flocs of all SBRs (Fig. 6.6), but the floc community structure varied widely. In $SBR_{aquaculture}$, the MaB-floc was dominated by cyanobacteria

(*Phormidium* sp.). In SBR_{manure}, unidentified coccal microalgal species dominated the floc, but filamentous cyanobacteria (*Phormidium* sp.) were also present.

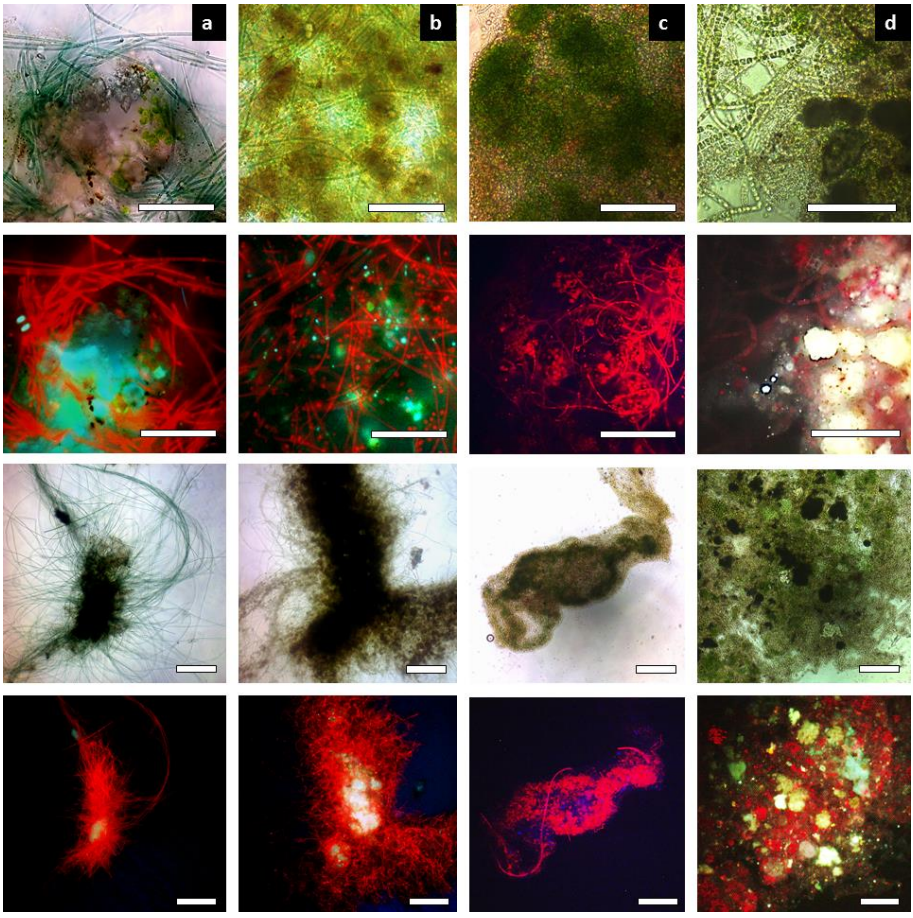


Fig. 6.6. *Light microscopy (1st and 3rd row) and fluorescence microscopy images (2nd and 4nd row) of MaB-flocs from SBRs treating wastewater of aquaculture (column a), manure treatment (column b), food-processing industry (column c) and chemical industry (column d)*

Depicted scale bars measure 50 µm in length. On fluorescence microscopy images, chlorophyll appears in red and crystals in yellow, blue or green.

In SBR_{food}, unidentified coccal microalgal species dominated the floc, but different filamentous cyanobacteria were also found (*Oscillatoria* sp. and *Phormidium* sp.). In $SBR_{chemical}$, a filamentous microalgae (*Stigeoclonium* sp. or similar looking specie) was the main photosynthetic microorganism in the MaB-flocs. Filamentous algae appear to be suitable candidates for cultivation in industrial

wastewaters because their size and structure make them relatively easy to harvest (Markou and Georgakakis, 2011). Light and fluorescence microscopy showed a strong presence of crystals in MaB-flocs from $SBR_{aquaculture}$ and SBR_{manure}, and to a lower extent in $SBR_{chemical}$ (Fig. 6.6.). This is reflected in their significantly lower VSS:TSS ratios of the latter three SBRs compared to SBR_{food} (Table 6.4). Since in SBR_{manure}, the TC removal rate of 0.027 ± 0.010 mg TC L^{-1} day^{-1} was around 40 % of the biomass productivity of 0.068 ± 0.067 g VSS L^{-1} day^{-1}. This is in line with the 36-65 % carbon content in microalgae (Van Den Hende et al., 2012a). Therefore, carbonate precipitation is not expected in SBR_{manure}. Further research, by using for example X-ray diffraction analyses, is needed to verify the nature of these crystals.

6.3.4. MaB-floc productivity in SBRs and dewatering

The type of wastewater did not significantly affect the average daily TSS productivities of MaB-flocs in SBRs (Table 6.4.). However, significant differences were found in the VSS:TSS ratio of MaB-flocs amongst SBRs. The highest average VSS:TSS ratio of MaB-flocs was obtained in SBR_{food}, being nearly double of that for SBRaquaculture and SBRmanure (Table 6.4.). This led to significantly different average VSS productivity in SBRfood (Table 6.4.). Compared to previous work with wastewater-fed MaB-flocs, the average VSS productivity in all SBRs of this study gave similar to high results (Table 6.5.).

MaB-floc photobioreactors were used in our experiments. However, MaB-floc SBRs on an industrial scale should be high-rate algal ponds (HRAPs). Without improvements, the MaB-flocs productivities in full-scale outdoor HRAPs are expected to be lower than lab-scale results, because of different temperature and light conditions outdoors, lower irradiated surface-to-volume ratio and maintenance downtime. The conversion of lab-scale productivities to outdoor conditions in HRAPs is highly dependent on the extrapolation factor applied, which is difficult to assess precisely without pilot outdoor experiments, and therefore sometimes non-realistically equated to 1. Nevertheless, our results seem promising. For example, applying an extrapolation factor of 0.3 would still lead to 48, 29, 98 and 53 ton VSS ha^{-1} $year^{-1}$ in a 0.40 m deep pond for MaB-floc SBRs treating wastewaters from aquaculture, manure, food-processing industry and chemical industry respectively.

***Table 6.5**. Comparison of this study with previous lab studies on wastewater treatment with microalgae and biomass production*

Wastewater	Species	Reactor	HRT (days)	Removal rate ($mg\ L_{reactor}^{-1}\ day^{-1}$)			Biomass productivity ($mg\ TS\ L_{reactor}^{-1}\ day^{-1}$)	Ref
				TCOD	TN	TP		
Domestic wastewaters	*Chorella vulgaris*	Batch 2L	n.a.[1]	2.5-42.5	6.63-1.60	0.05-3.00	56-195[2]	Cabanelas et al., 2013
Domestic wastewaters	*C. vulgaris* + AS[3]	Batch 2L	n.a.	10.0-10.8	2.70-2.80	0.39	69-87[2]	Cabanelas et al., 2013
SAnMBR effluent[4] + CO_2	Algae mix	SCR[5] 8L	2	n.d[6]	19.5 ± 5[7]	7.3 ± 1.6	234 ± 32	Ruiz-Mart. et al., 2012
Piggery, anaerobic effluent	*Scenedesmus* sp.	SCR[5] 1L	n.d.	n.d.	19.2[7]	n.d.	213	Park et al., 2010a
Secondary effluent	*Phormidium* sp.	Batch 5L	n.a.	n.d.	3.66 ± 0.17	0.56 ± 0.07	(2.71 ± 0.7)[8]	Su et al., 2012a
Sewage	*Chorella* sp.+AS[3]	CSTR 30L	1.5	417	57	5.7	85	Gutzeit, 2006
Sewage	*Chorella* sp+AS[3]	CSTR 30L	2	412	67	10.4	41	Gutzeit, 2006
Sewage	*Chorella* sp+AS[3]	CSTR 300L	5-7.5	416	53	4.8	28	Gutzeit, 2006
Synthetic wastewaters	MaB-flocs	SBR 1.5L	0.67	154-320[9]	3.3 - 20.4[9]	n.d.	n.d.	VD Hende et al., 2011b
Sewage + 0.6 L h^{-1} flue gas	MaB-flocs	SBR 4L	0.67	n.d.	40.5 ± 5.5	2.26 ± 0.93[10]	181 ± 122[11]	VD Hende et al., 2011a
Aquaculture	MaB-flocs	SBR 4L	2	65 ± 6	9.8 ± 1.8	1.56 ± 0.08	109 ± 30[11]	This study
Manure treatment	MaB-flocs	SBR 4L	2	30 ± 5	4.5 ± 1.0	0.11 ± 0.01	68 ± 67[11]	This study
Food UASB effluent	MaB-flocs	SBR 4L	2	256 ± 37	29.1 ± 14.2	2.74 ± 0.90	223 ± 90[11]	This study
Chemical industry	MaB-flocs	SBR 4L	2	157 ± 40	18.1 ± 3.1	0.31 ± 0.34	122 ± 140[11]	This study

[1] Not applicable; [2] Suspended solids; [3] Activated sludge; [4] Submerged Anaerobic Membrane Bioreactor effluent; [5] Semi-continuous reactor without biomass settling phase; [6] No data; [7] $N\text{-}NH_4^+$ removal; [8] Areal productivity in $g\ m^{-2}\ day^{-1}$; [9] Range of average removals of sCOD and $N\text{-}NO_3^-$ of various wastewaters; [10] $P\text{-}PO_4^{3-}$; [11] VSS.

Microalgae harvesting is one of the main bottlenecks of microalgal technology for wastewater treatment (Udom et al., 2013; Uduman et al., 2010). In general, microalgae are harvested by a first upconcentrating step producing a microalgal slurry of 2-7 % TSS, for example by flocculant addition, followed by a second dewatering step producing a microalgal paste of 15-25 % TSS, for example by centrifugation (Uduman et al., 2010).

In this study, MaB-flocs were harvested in one single step via a filter press with a large pore size of around 200 µm. No flocculants were added and the MaB-flocs did not need to settle prior to dewatering. Based on lifecycle assessment analyses, belt presses are a recommended algae dewatering technology if high solid contents are desired (Udom et al., 2013). This filter press dewatering was successful and stable for $SBR_{aquaculture}$ and SBR_{manure}, resulting in algal cakes with a high dry matter content and high MaB-floc recovery (Table 6.4.). For SBR_{food}, lower but still adequate dewatering and biomass recoveries were obtained (Table 6.4.). This successful dewatering was due to relatively large flocs and to the dominance of filamentous cyanobacteria in these MaB-flocs (Fig. 6.6.). In contrast, MaB-flocs in $SBR_{chemical}$ could only be dewatered with this filter press at the end of the SBR run when the flocs became large enough in size to remain on the 200 µm filter screen. The results for MaB-floc recovery in $SBR_{chemical}$ were not stable and lower on average compared to results for all other SBRs (Table 6.4.). A lower pore size of the filter press would be needed to obtain more stable results, but will also decrease the flow-through rate and increase the dewatering costs.

Overall, the type of wastewater largely affected the MaB-floc architecture and suitability for filter press dewatering. Since outdoor conditions might also change the MaB-floc community structure and MaB-floc architecture, these successful lab-scale results should be confirmed with outdoor grown MaB-flocs. Furthermore, the filter pore size, pressure and pressing time should be optimised at a pilot scale.

6.4. Conclusions

Settling MaB-flocs were developed and used in SBRs to treat wastewater of various (agro-)industrial sectors, specifically aquaculture, manure treatment, food-processing industry and chemical industry. The wastewater type strongly affected the nutrient removal, effluent quality and floc characteristics. Very low phosphorous concentrations in manure and aquaculture effluent highlight the potential of MaB-floc SBRs for phosphorous polishing. High MaB-floc biomass productivities were obtained and MaB-flocs were successfully dewatered by filter press with high biomass recovery. Although for some wastewaters further optimisation is needed to reach current discharge norms, our results demonstrate the wide applicability of dual-purpose MaB-floc SBRs.

Outdoor experiments in raceway ponds during both cold and warm seasons are needed to confirm these lab-scale results. Amongst the wastewaters tested in this study, aquaculture wastewater and UASB effluent from food-processing industry seem the most suitable candidates for treatment in a MaB-floc SBR. Therefore, it was decided to design, construct and operate an outdoor MaB-floc open pond to treat wastewater from aquaculture in 2013 and UASB effluent from food-processing industry in 2014.

6.5. Acknowledgements

Thiswork was supported by the INTERREG IVB NWE Programme, the Flemish Government, Province West-Flanders, University College Howest and UGent (LIWET) within the EnAlgae project. The authors thank the companies Alpro, BASF and Innova Manure, and the research institute Inagro for providing wastewater, on site collected microalgae and discussion of results; H. Vervaeke (Howest/UGent) for installing the PLC steering and data logging; J. Van Wichelen (UGent) for the identification of microalgae; J. Coppens (UGent) for proof-reading the manuscript.

Size matters.

Picture on previous page:

Clown Valle, one of my sisters, trying to dive in a too small algae reactor.

CHAPTER 7

Up-scaling of treatment of aquaculture wastewater in MaB-floc SBRs: from lab reactors to an outdoor raceway pond

Abstract

Sequencing batch reactors with microalgal bacterial flocs (MaB-floc SBRs) are a novel approach for photosynthetic aerated wastewater treatment based on bioflocculation. To assess their technical potential for aquaculture wastewater treatment in Northwest Europe, MaB-floc SBRs were up-scaled from indoor photobioreactors of 4 L, 40 L and 400 L to an outdoor raceway pond of 12 m^3. Scale-up decreased the nutrient removal efficiencies with a factor 1-3 and volumetric biomass productivities with a factor 10-13. Effluents met current discharge norms, except for nitrite and nitrate. Flue gas sparging was needed to decrease the effluent pH. Outdoor MaB-flocs showed enhanced settling properties and an increased ash and chlorophyll *a* content. Bioflocculation enabled successful harvesting by gravity settling and dewatering by filtering at 150-250 µm. Optimisation of nitrogen removal and biomass valorisation are future challenges towards the industrial implementation.

Chapter redrafted after:

Van Den Hende. S., Beelen, V., Bore, G., Boon, N., Vervaeren, H.. Up-scaling of treatment of aquaculture wastewater in MaB-floc SBRs: from lab reactors to an outdoor raceway pond. Submitted.

7.1. Introduction

In recent years, intensive aquaculture has developed all over the world, bringing with it a whole new set of environmental concerns, primarily concentrated around the issue of waste disposal (Martins et al., 2010; Richmond, 2004). Approximate 75 % of the aquaculture feed remains as nitrogen and phosphorous in the wastewater (Crab et al., 2007). During the past century, major efforts have been undertaken to remove these nutrients from aquaculture wastewater to obtain a satisfactory effluent quality that does not result in eutrophication of natural recipient ecosystems and/or that enables water recycling (Váradi et al., 2009; Crab et al., 2007). Nowadays, global warming and depletion of resources such as fossil energy, fresh water and phosphorous, mandates urgent efforts to redesign conventional wastewater treatment systems towards energy efficiency and nutrient recovery (Holmgren, 2002). Microalgae could play a key role in this redesign. Being photosynthetic microorganisms, they consume CO_2, lower the need for mechanical aeration by providing oxygen, scavenge resources from the wastewater (C, N and P) and convert solar energy into biomass (Van Den Hende et al., 2011a).

A key factor determining the economic viability of microalgae systems for wastewater treatment is the cost-effective separation of microalgae from the treated water (Udom et al., 2013; Park et al., 2011a). In this regard, a novel approach based on bioflocculation of microalgae was developed. Microalgal bacterial flocs in sequencing batch reactors (MaB-floc SBRs) settle by gravity (Van Den Hende et al., 2011a). After this settling of MaB-flocs in the reactor, biomass-free effluent can be discharged. In this way, no expensive biomass removal techniques are needed for effluent discharge. Moreover, as the MaB-floc biomass is harvested, gravity settling provides a first concentration step of the MaB-floc biomass fraction.

On lab-scale, MaB-floc SBRs showed promising results for the treatment of pikeperch culture wastewater and concomitant biomass production (Chapter 6). Furthermore, MaB-floc biomass produced during these lab-scale experiments was efficiently harvested by a 200 µm pore filter press. On industrial scale, MaB-floc SBRs should be operated in outdoor raceway ponds, because raceway ponds tend to be the cheapest, the easiest to install and to operate, and the most durable microalgae culture systems (Richmond, 2004). In these shallow (0.4 m) algae raceway ponds, adequate nutrient removal rates are of importance because they determine the reactor

volume and consequently the needed land area. Moreover, adequate MaB-floc settling and removal are needed to obtain a biomass-free effluent and to reach the target effluent norms. For biomass valorisation, the dominant presence of microalgae in MaB-flocs, the biomass productivity and its dewatering are of interest. Concerning up-scaling, lab-scale results should not be simply extrapolated to industrial outdoor scale. For example, extrapolation of data from treatment of pikeperch wastewater in a MaB-floc SBR of 4 L (Chapter 6), would result in an unrealistic biomass productivity of 160 ton volatile suspended solids (VSS) ha^{-1} $year^{-1}$. Up-scaling to the outdoors brings along its specific outdoor operation conditions with respect to temperature (diurnal and seasonal fluctuations), light (intensity, spectral quality, photoperiod), reactor dimensions (depth, irradiated surface:reactor volume ratio, buffering), turbulence, contamination risk (dirt, algae grazers), harvesting equipment and wastewater composition variability (Richmond, 2004). Moreover, outdoor operation might require pH control by flue gas injection to reach the target discharge norm (< 9.5) and to avoid inhibition of activity of aerobic heterotrophic bacteria (optimum 8.3) (Park and Craggs, 2010; Oswald, 1988).

To assess the technical feasibility of outdoor MaB-floc SBRs for treatment of pikeperch wastewater in Northwest Europe, MaB-floc SBRs were up-scaled from indoor lab-scale reactors (4 L, 40 L, 400 L) to an outdoor raceway pond (12 m^3). The key factors governing the technical feasibility of MaB-floc SBRs are evaluated: (1) wastewater treatment (nutrient removal, effluent quality, need for flue gas sparging), (2) MaB-floc characteristics (floc settling, chlorophyll and ash content), (3) biomass productivity, and (4) MaB-floc harvesting by gravity settling followed by filtering at 150-250 µm. Moreover, by comparing the reactor performances, a first assessment of the conversion factors to translate lab-scale results to outdoor scale is provided.

7.2. Materials and methods

7.2.1. Indoor SBR of 40 L

The SBR with a working volume of 40 L was operated with a hydraulic retention time (HRT) of 2 days, referred to as 40L_T2 (Table 7.1; Fig. 7.1.a). This reactor consisted of an open tank which was illuminated by one halogen lamp of 500 W (Silon CE-82-Y, Hong-Kong) and 3 halogen lamps of 20 W (Lunaqua 3, Oase, Germany). The reactor was heated to 25 °C (HT 75W, TETRA, Germany).

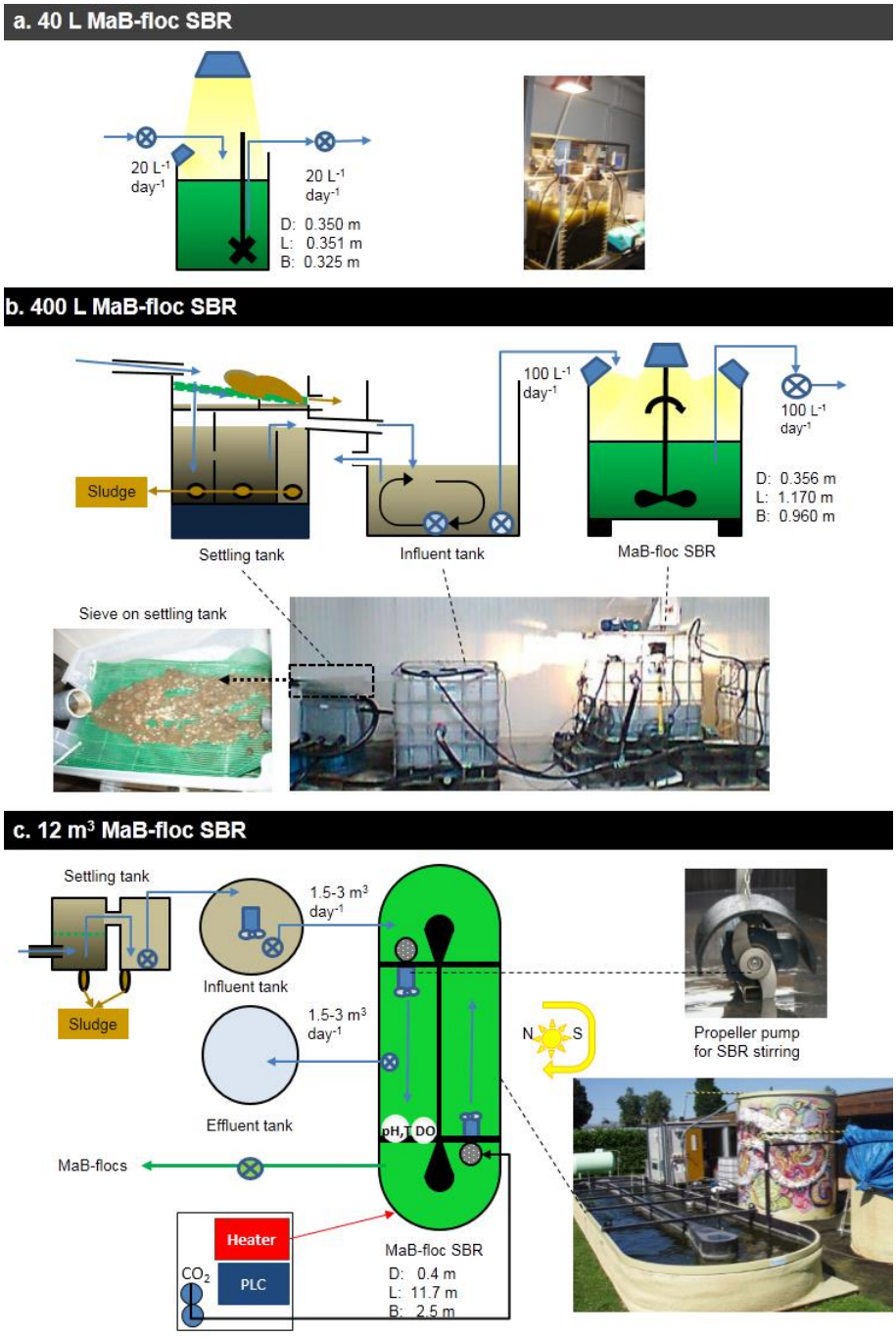


Fig. 7.1. *Set-up of lab-scale (a, b) and outdoor (c) MaB-floc SBRs treating aquaculture wastewater*

Table 7.1. *Operation modes of MaB-floc SBRs of 40 L, 400 L and 12 m³*

Reactor[1]	Time period	Effl. out (L)	Infl. in (L)	Stirring (rpm)	FGFR (L h⁻¹)	PPFD (µmol PAR photons m⁻² s⁻¹)
40L_T2	**2012/09/11 – 2012/11/21**					
	8.00 - 8.45	20	0	0	0	0
	8.45 - 9.00	0	}	0	0	0
	9.00 - 9.30	0	20	100-210	0	139 ± 33[2]
	9.30 - 21.00	0	0	100-210[2]	0	139 ± 33[2,3]
	21.00 - 9.00	0	0	0	0	0
400L_T4_0.75	**2012/11/30 – 2013/01/14**		}			
400L_T4_0.50	**2013/01/14 – 2013/03/08**					
	7.00 - 9.00	100	0	0	0	0
	9.00 - 9.15	0	100	0	0	89 ± 36[4]
	9.15 - 21.00	0	0	53	0	89 ± 36[4]
	21.00 - 22.00	0	0	53	0	0
	22.00 - 7.00	0	0	0	0	0
12M_1_T8_F0[5]	**2013/01/24 - 2013/02/07**					
Start-up	8.30 - 9.00	1500	0	0	0	Outdoor
	9.00 - 9.20	0	1500	0	0	Outdoor
	9.20 - 18.00	0	0	1200	0	Outdoor
	18.00 - 9.00	0	0	0	0	Outdoor
12M_2_T4_F0[5]	**2013/02/07 - 2013/03/27**					
Start-up	8.30 - 9.00	3000	0	0	0	Outdoor
	9.00 - 9.30	0	3000	0	0	Outdoor
	9.30 - 18.00	0	0	1200	0	Outdoor
	18.00 - 9.00	0	0	si[6]	0	Outdoor
12M_3_T4_F0	**2013/03/27 – 2013/04/17**					
	7.30 - 8.00	3000	0	0	0	Outdoor
	8.00 - 8.30	0	3000	0	0	Outdoor
	8.30 - 20.30	0	0	1200	0	Outdoor
	20.30 - 7.30	0	0	si[7]	0	Outdoor
12M_4_T4_F3	**2013/04/17 - 2013/04/27**					
	7.30 - 8.00	3000	0	0	0	Outdoor
	8.00 - 8.30	0	3000	0	0	Outdoor
	8.30 - 20.30	0	0	1200	200[10]	Outdoor
	20.30 - 7.30	0	0	si[8]	0	Outdoor
12M_5_T8_F3	**2013/04/27 - 2013/05/09**					
	7.30 - 8.00	1500	0	0	0	Outdoor
	8.00 - 8.30	0	1500	0	0	Outdoor
	8.30 - 20.30	0	0	1200	200 [10]	Outdoor
	20.30 - 7.30	0	0	si[8]	0	Outdoor
12M_6_T8_F0	**2013/05/09 - 2013/06/06**					
	7.30 - 8.00	1500	0	0	0	Outdoor
	8.00 - 8.30	0	1500	0	0	Outdoor
	8.30 – 21.00	0	0	1200	0	Outdoor
	21.00 - 7.30	0	0	si[9]	0	Outdoor
12M_7_T4_F0	**2013/06/06 - 2013/07/22**					
	7.20 - 8.00	3000	0	0	0	Outdoor
	8.00 - 8.45	0	3000	0	0	Outdoor
	8.45 - 22.30	0	0	1200-1500	0	Outdoor
	22.30 - 7.20	0	0	si[9]	0	Outdoor
12M_8_T4_F5	**2013/07/22 - 2013/09/12**					
	7.20 - 8.00	3000	0	0	0	Outdoor
	8.00 - 8.45	0	3000	0	0	Outdoor
	8.45 - 22.30	0	0	1400-1500	300 [10]	Outdoor
	22.30 - 7.20	0	0	si[9]	0	Outdoor

[1] Names of indoor reactors and periods are composed as 'reactor volume in L'L_T'HRT in days'_'TSS in g L^{-1} after harvest' ; names of operation periods of 12 m^3 reactor are composed as 'reactor volume in m^3'M_'Operation period'_T'HRT in days'_F'flue gas flow rate in L min^{-1}'; [2] Average PFD measured on 30 locations on the reactor water surface; [3] Light and stirring were turned off from 15.00-15.15 to cool

down lamp and motor respectively; [4] Average PPFD measured on 56 locations on the reactor water surface; [5] Start-up period with MaB-floc density < 0.50 g TSS L^{-1}; [6] Short interval (si) stirring at1200 rpm during 3.00-3.05, 21.00-21.05; [7] Short interval stirring at1200 rpm during 0.00-0.05, 4.00-4.05, 22.00-22.05; [8] Short interval stirring at 1200 rpm during 4.00-4.05, 22.00-22.05; [9] Short interval stirring at 1200 rpm during 1.00-1.30, 4.00-4.30; [10] Flue gas injection at this flue gas flow rate when pH > 9.0.

Peristaltic pumps were used for influent feeding and effluent withdrawal (both Watson Marlow, USA). The reactor was equipped with an overhead stirrer (100 rpm during days 1-2, 130 rpm during days 2-12, and 210 rpm during days 13-97) (Heidolph RZR 2020, Germany). No mechanical aeration of the reactor was performed. The aquaculture wastewater was drum filter effluent (30 µm) from indoor pikeperch culture (Inagro, Belgium), as earlier described (Chapter 6). This wastewater was sieved at 3 mm and stored at 4 °C until use. MaB-flocs from previous experiment in a 4 L SBR treating pikeperch culture wastewater (Chapter 6) were up-scaled to 3 SBRs of 5 L during 2 months (no data shown) and used as inoculum for 40L_T2. From day 9 till 68, influent and effluent were sampled weekly whereas MaB-flocs were sampled 1-2 times a week. During days 69-97, 40L_T2 was operated to produce MaB-floc inoculum for up-scaling. MaB-flocs were harvested 1-2 times a week to maintain 1 g total suspended solids (TSS) L^{-1} and were stored at 4 °C to use as inoculum.

7.2.2. Indoor SBR of 400 L

The SBR with a working volume of 400 L was operated with a HRT of 4 days, referred to as 400L_T4 (Table 7.1.; Fig. 7.1.b). The reactor was a plastic open tank illuminated by one halogen lamp of 500 W (Silon CE-82-Y, Hong-Kong) and 10 halogen lamps of 20 W (Lunaqua 3, Oase, Germany). The reactor was operated indoors (14-23 °C; Inagro, Roeselare) without heating. A centrifugal pump was used for influent feeding (Eco 3000 Aquarius Universal 30 W, Oase, Germany) controlled by a level regulator (TKZC4000F REKA 2000, MAC3 Water Systems, Italy). A peristaltic pump (520SN/R2, Watson Marlow, USA) was used for daily effluent withdrawal after the MaB-floc settling phase at night. An overhead stirrer was used for mixing (53 rpm; T200020-0710-0.55 kW, BCI Electromotoren nv, Belgium). No mechanical aeration of the reactor was performed. Pikeperch culture wastewater (same origin as for 40L_T2) was continuously fed to an influent tank after sieving at 4 mm and subsequent settling (Fig. 7.1.b). Fish feed particles and faeces were removed 2-3 times a week from the sieve and settling compartments. The influent

buffer tank was mixed every 0.25 h h-1 with a pump (Baseline, Maxeda DIY bvba, The Netherlands) to avoid sludge accumulation. The 400L_T2 reactor was inoculated with MaB-flocs at start (25.80 g TSS, 19.75 g VSS), at day 7 (18.80 g TSS, 14.85 g VSS) and at day 18 (21.68 g TSS, 15.74 g VSS). MaB-flocs were frequently harvested to maintain 0.75 g TSS L^{-1} during period 1 (day 19-45, referred to as 400L_T4_0.75) and 0.50 g TSS L^{-1} during period 2 (day 46-98, referred to as 400L_T4_0.50) (Table 7.1.). Harvested MaB-flocs were stored at 4 °C until further use as inoculum. Twice a week, MaB-flocs, influent and effluent were sampled.

7.2.3. Outdoor SBR of 12 m^3

The SBR with a working volume of 11.959 m^3 was a raceway pond , referred to as 12M (Fig. 7.1.c). This raceway was operated outdoors (50°54' 212'' North, 3° 7'571'' East; Inagro, Roeselare, Belgium). To enhance start-up during winter, the reactor was operated at a minimum temperature of 12 °C by a heating system consisting of warm water tubes and gas boiler (Bulex, Belgium). Two propeller pumps (DRENO, Italy) stirred the raceway. Effluent was withdrawn with a submerged pump (Industrial pump system bvba, Belgium). The outdoor influent buffer tank was discontinuously stirred with a propeller pump (Dreno, Monselice, Italy; 6-7 periods of 0.08-0.75 h day^{-1} including during reactor feeding) to avoid sludge accumulation while wasting excess influent. Wastewater from pikeperch culture (same origin as 40L_T2) was mechanically pre-treated in an indoor settling tank. The first compartment removed particles larger than 1.2 mm by a vertical sieving screen. The second compartment removed settling and floating sludge. From here the water flowed to an indoor buffer tank and was pumped (emerged pump EUS, EVAK Taichung, Taiwan) to an outdoor influent buffer tank (HRT of all tanks was max. 3 days). Biweekly, excess fish feed and sludge were removed from the indoor tanks.

The reactor was operated for 231 days from winter to summer with 8 different operation periods (Table 7.1.). Names of reactor operation periods are composed as 'reactor volume in m^3'M_'operation period'_T'HRT in days'_F'flue gas flow rate in L min^{-1}'. During start-up, the HRT of 8 days in period 1 (12M_1_T8_F0) was decreased to 4 days in period 2 (12M_2_T4_F0). At the start of period 3, the MaB-floc density in the reactor reached 0.5 g TSS L^{-1} and harvesting was started to maintain 0.5 g TSS L^{-1} (12M_3_T4_F0). Flue gas was sparged during period 4

(12M_4_T4_F3) and 5 (12M_5_T8_F3) containing 214 ± 4 g CO_2 Nm^{-3}, 383 ± 8 mg NO Nm^{-3}, 572 ± 11 mg Nm^{-3} SO_2 (Lindegas, Belgium). Due to a temporary lack of wastewater volume, the HRT was increased to 8 days during period 5 (12M_5_T8_F3) and 6 (12M_6_T8_F0). To demonstrate the need for flue gas sparging, no flue gas was sparged during period 7 (12M_7_T4_F0) and flue gas containing 89 ± 2 g CO_2 Nm^{-3} (Lindegas, Belgium) was sparged during period 8 (12M_8_T4_F5). During start-up, 12M was inoculated with 217, 38, 18, 46, 22, 134, 49 g TSS and 148, 32, 14, 33, 16, 102, 39 g VSS of MaB-flocs at day 1, 5, 8, 15, 18, 26, 35 and 44 respectively; being in total 0.044 g TSS $L_{reactor}^{-1}$ and 0.032 g VSS $L_{reactor}^{-1}$. The reactor DO (Visiferm DO probe, Hamilton, Belgium), reactor pH and reactor temperature ($T_{reactor}$) (201020/51-18-04 pH Pt 100 probe, Jumo, Germany), photosynthetic photon flux density (PPFD) (400-700 nm, LI-190 PAR light sensor, Li-Cor, USA), ambient temperature ($T_{ambient}$) (E+E Elektronik, Austria), reactor level (PEPPERL+FUCHS ultrasonic sensor, Belgium), status of pumps, and flue gas flow rate (FGFR) (Mass-view CO_2 sensor, Mass-flow, Belgium) were logged minimum every 2 minutes. MaB-flocs, influent and effluent were sampled 2-3 times a week.

7.2.4. Harvesting of MaB-flocs

Harvesting of MaB-flocs from 12 M consisted of 2 steps: (1) concentration by settling and (2) dewatering by natural filtering followed by press filtering. In the first step, MaB-floc liquor was pumped with a flexible impeller pump (EP Midex 1400 L h^{-1}, Liverani, Italy) from the raceway pond into a 1 m^3 settling tank. After 1 h settling, supernatant (0.8 m^3) was pumped back in the raceway pond. In the second step, the remaining supernatant and settled MaB-flocs (0.2 m^3) were pumped in a linen filter bag (150-250 µm pore size; Lampe, Belgium). During pumping, this MaB-floc containing filter bag was first dewatered by gravity, resulting in a MaB-floc slurry and gravity filtrate. Thereafter, the slurry containing filter bag was further dewatered by hydropress (4 bar; Enotecnica Pillan, Italy) obtaining a MaB-floc cake and press filtrate. MaB-floc reactor liquor of the settling tank before settling, supernatant of the settling tank after settling, gravity filtrate and press filtrate were analysed for VSS and TSS. MaB-floc losses during harvesting were calculated as the percentage of the total initial biomass in the buffer tank lost by settling, gravity filtering or press filtering. MaB-floc cakes were analysed for total solids (TS) and volatile solids (VS).

7.2.5. Analytical protocols

Water samples were analysed for pH, turbidity, total organic carbon (TOC), total inorganic carbon (TIC), total carbon (TC), total nitrogen (TN), total phosphorous (TP), chemical oxygen demand (COD) and biological oxygen demand (BOD_5), and MaB-flocs samples were analysed for TSS, VSS, TS, VS, chlorophyll *a* (Chl*a*), physiological condition ($A664_b$:$A665_a$), autotrophic index (AI), diluted (dSVI), and by light and fluorescence microscopy, according to Chapter 6. Electron conductivity (EC) was measured on water samples (K610, Consort bvba, Belgium). Filtered (0.2 µm RC 20/25, Chromafil, Germany) influent and effluent of 400L_T4_0.50 and 12M were analysed for NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} with spectrophotometric kits (Hach Lange, Belgium). Removal efficiencies and rates were calculated from daily influent and effluent values.

7.2.6. Statistics

Statistical analyses were performed using PASW Statistics 17.0 software (SPSS Inc, USA). Normal distribution of data was screened with a Shapiro-Wilk test and homogeneity of variances with a Levene's test. In case of normal data distribution and homogeneity of variances, significant differences were analysed by a one-way ANOVA and a Tukey's post-hoc test ($p < 0.05$). Otherwise, Kruskal-Wallis followed by a Mann-Whitney post-hoc test was used ($p < 0.05$). Averages are given with standard deviations. Correlations were quantified with non-parametric Spearman's r_s (two-tailed significance; $p < 0.005$).

7.3. Results and discussion

7.3.1. Wastewater treatment

7.3.1.1. MaB-floc SBR of 40 L

In reactor 40L_T2, the applied daily PPFD (dPPFD) of 17 mmol PAR photons $L_{reactor}^{-1}$ day^{-1} was only 47 % of the average PPFD outdoors. Nevertheless, a significant decrease in turbidity, COD, BOD_5 and TOC in the MaB-floc SBR was observed (Fig. 7.2.a). This resulted in a high removal rate (RR) and removal efficiency (RE) of these parameters (Table 7.2.; Table 7.3.). In contrast, the REs for TIC and EC were negative and showed strong variations (Fig. 7.3.a; Table 7.3.). The pH

significantly increased from 7.31 ± 0.18 to 7.81 ± 0.11 (Fig. 7.3.a). The obtained REs were sufficient to reach the current discharge norms for this wastewater for pH (6.0-9.5), COD (125 mg L^{-1}), BOD_5 (25 mg L^{-1}), TN (99.8 mg L^{-1}; sum of Total Kjeldahl N, N-NO_3^- and N-NO_2^-) and TP (24 mg L^{-1}) (Fig. 7.2.a; Fig. 7.3.a; Fig. 7.4.a). Per g VSS produced, 1.99 ± 0.62 g COD, 1.08 ± 0.24 g BOD_5, 0.52 ± 0.17 g TOC, 0.35 ± 0.22 g TC and 0.22 ± 0.07 g TN were removed from the wastewater, and 16 ± 0.17 g TIC was net produced. This means that for each mol TN removed, 1.52 ± 1.43 mol TC and 2.38 ± 1.33 mol TOC was removed. The latter values are low compared to the TC:TN ratio of microorganisms of approximately 6 (Geider and La Roche, 2002). This suggests that the nitrogen removal could not be due to biomass growth only. More research is needed to confirm whether denitrification in anoxic microniches of the flocs, as in activated sludge (Schramm et al., 1999), was an additional mechanisms for N removal.

7.3.1.2. MaB-floc SBR of 400 L

To provide MaB-floc inoculum for the 12 m^3 reactor, harvesting was increased in the 400 L reactor, and the MaB-floc density was decreased from 0.75 g TSS L^{-1} in period 1 (400L_T4_0.75) to 0.50 g TSS L^{-1} in period 2 (400L_T4_0.50). During period 2, the influent turbidity was increased with a factor 3 and the influent COD, BOD_5, TOC, TN and TP concentrations were doubled compared with period 1 (Fig. 7.2.b; Fig. 7.4.b). The dPPFD of 11 mmol PAR photons $L_{reactor}^{-1}$ day^{-1} was only 31 % of the average dPPFD outdoors (Fig. 7.5). Nevertheless, during both operation periods, adequate RRs and REs were obtained (Table 7.2.; Table 7.3.). The effluent reached the current discharge norms for pH, COD, BOD_5, TN (except for 1 sample) and TP (Fig. 7.2.b. Fig. 7.3.b; Fig. 7.4.b). In spite of the large variations of the influent turbidity, the effluent turbidity was rather stable. This might be due to the sequenced settling and/or dominance of a *Phormidium* sp. in the MaB-flocs. These filamentous cyanobacteria can release turbidity removing bioflocculants (Bar-Or and Shilo, 1987), and acted like a filtering net during MaB-floc settling. The TIC was only significantly removed during period 2 (Fig. 7.3.b). During both operation periods, no significant decrease in EC and increase in pH was observed (Fig. 7.3.b). Therefore, flue gas sparging was not needed for pH control.

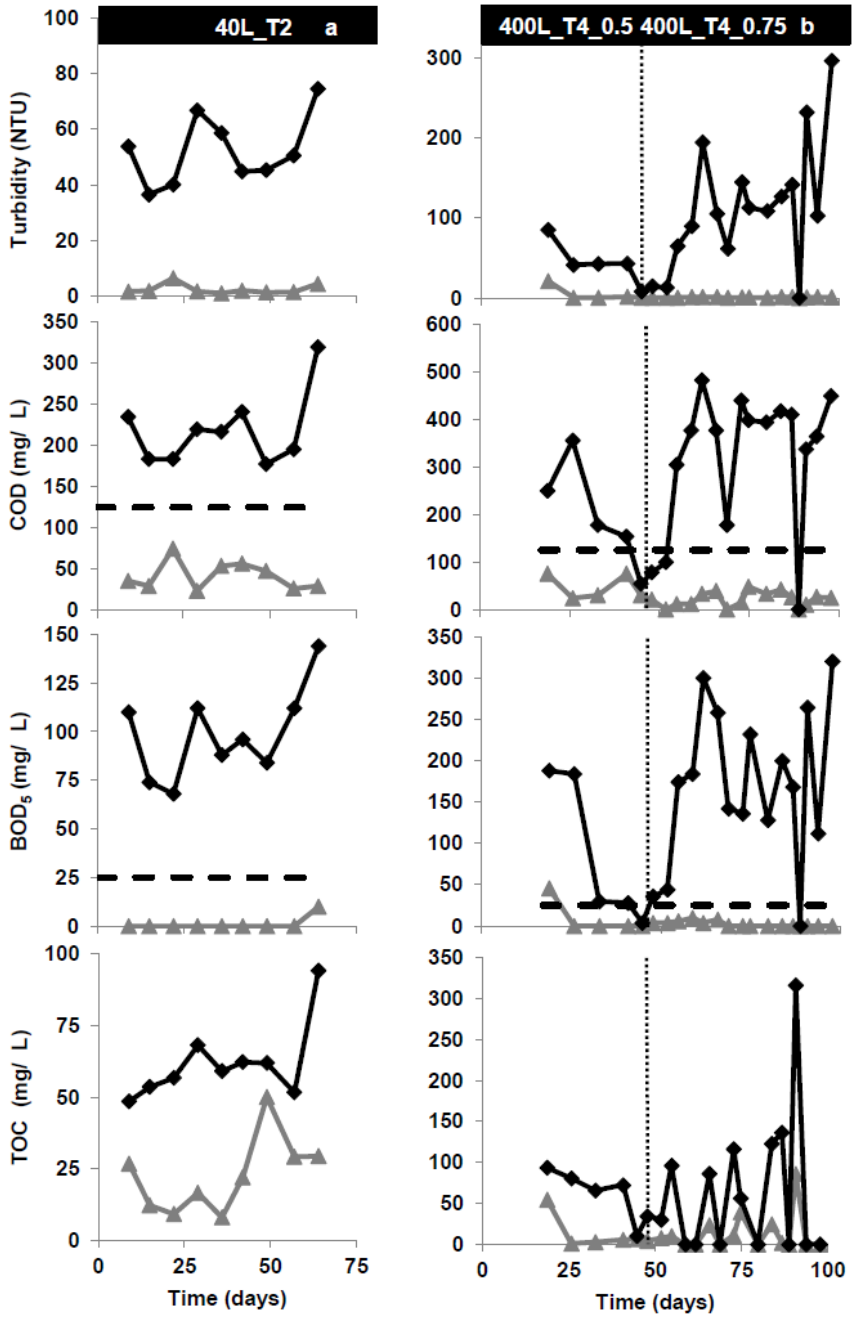
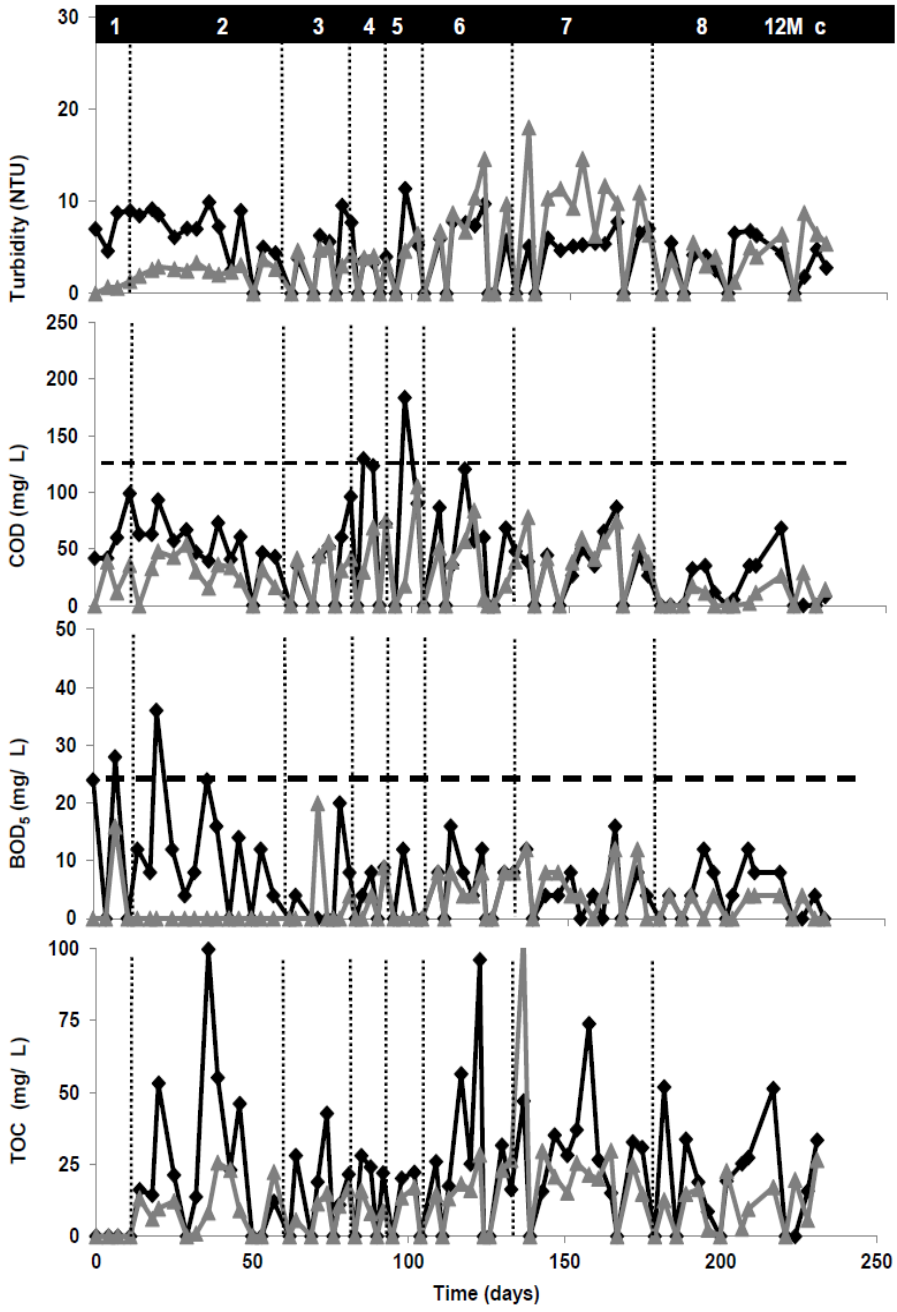


Fig. 7.2. *Turbidity, COD, BOD and TOC of influent (♦) and effluent (▲) of aquaculture wastewater treating MaB-floc SBRs of 40 L (a), 400 L (b) and 12 m^3 (c, on next page)*

Discharge norms (- - - - -) according to current (2013/09) environmental permit for this wastewater



(Figure caption on previous page)

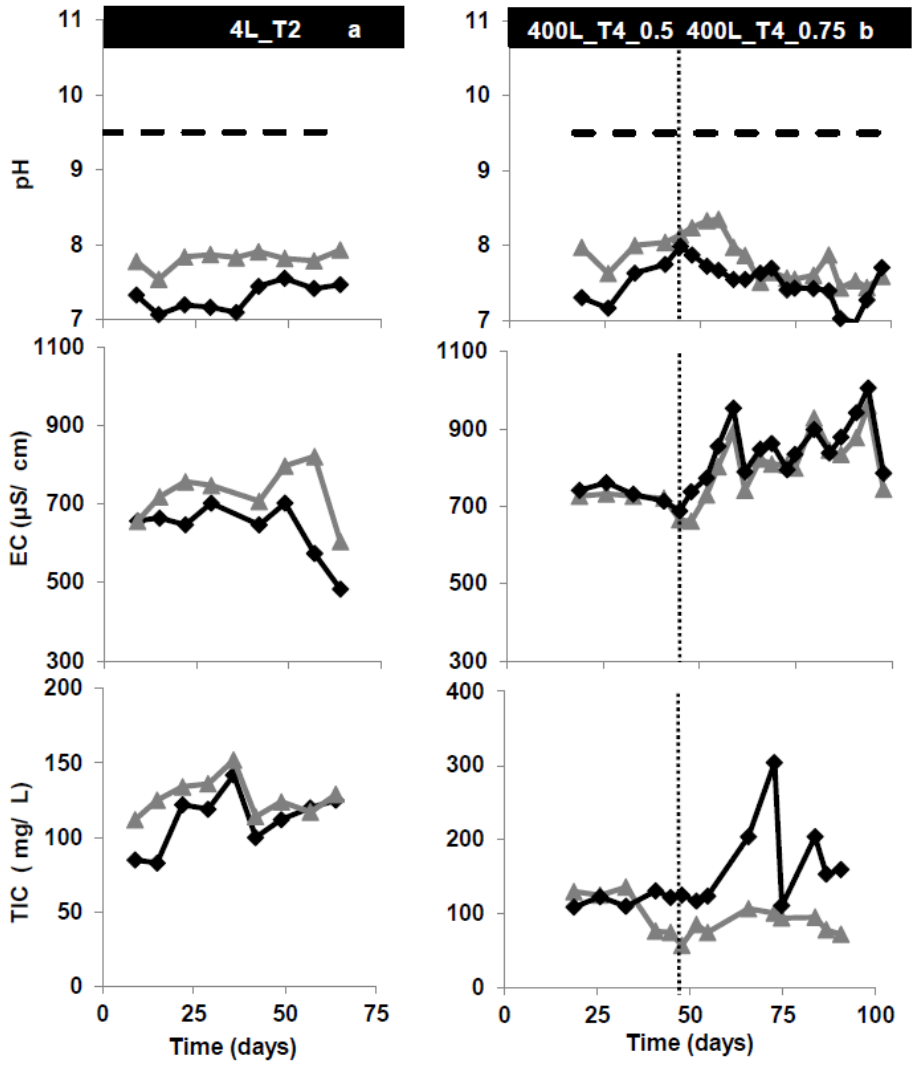
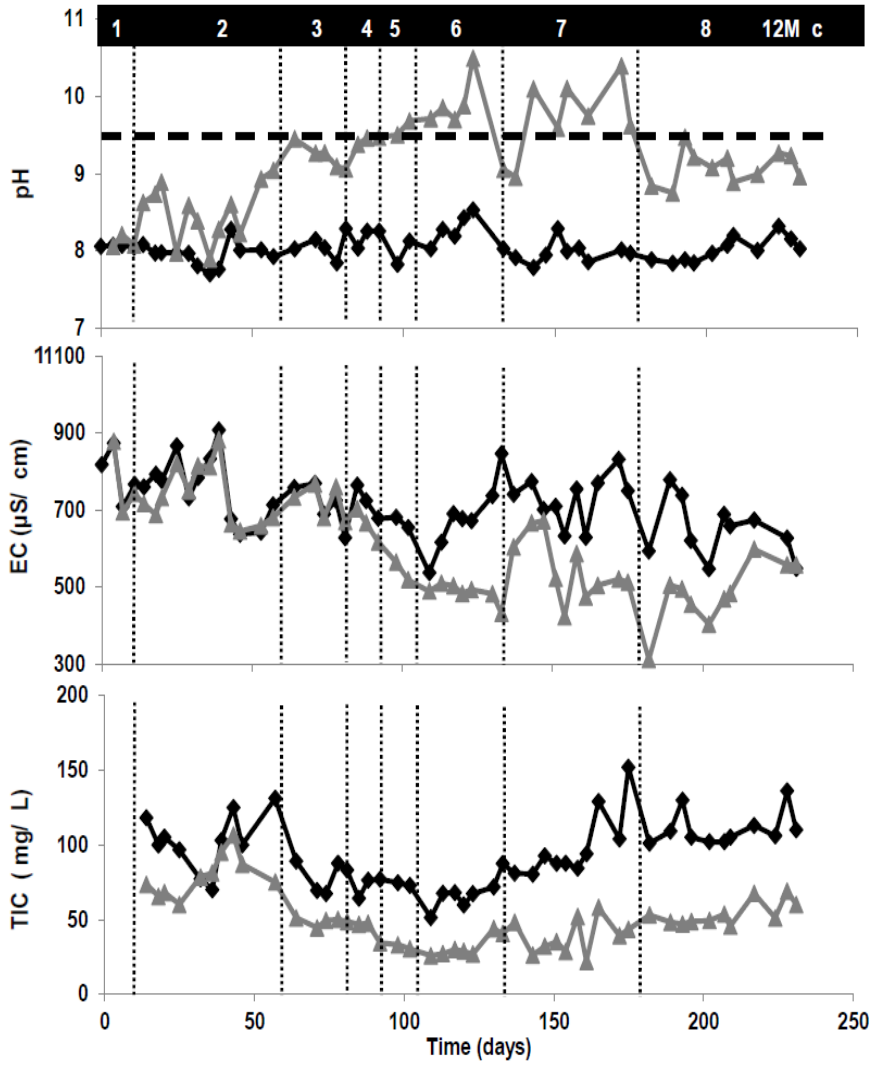


Fig. 7.3. *TIC, EC and pH of influent (♦) and effluent (▲) of aquaculture wastewater treating MaB-floc SBRs of 40 L (a), 400 L (b) and 12 m^3 (c, on next page)*
Discharge norms (- - - - -) according to current (2013/09) environmental permit for this wastewater.



(Figure caption on previous page)

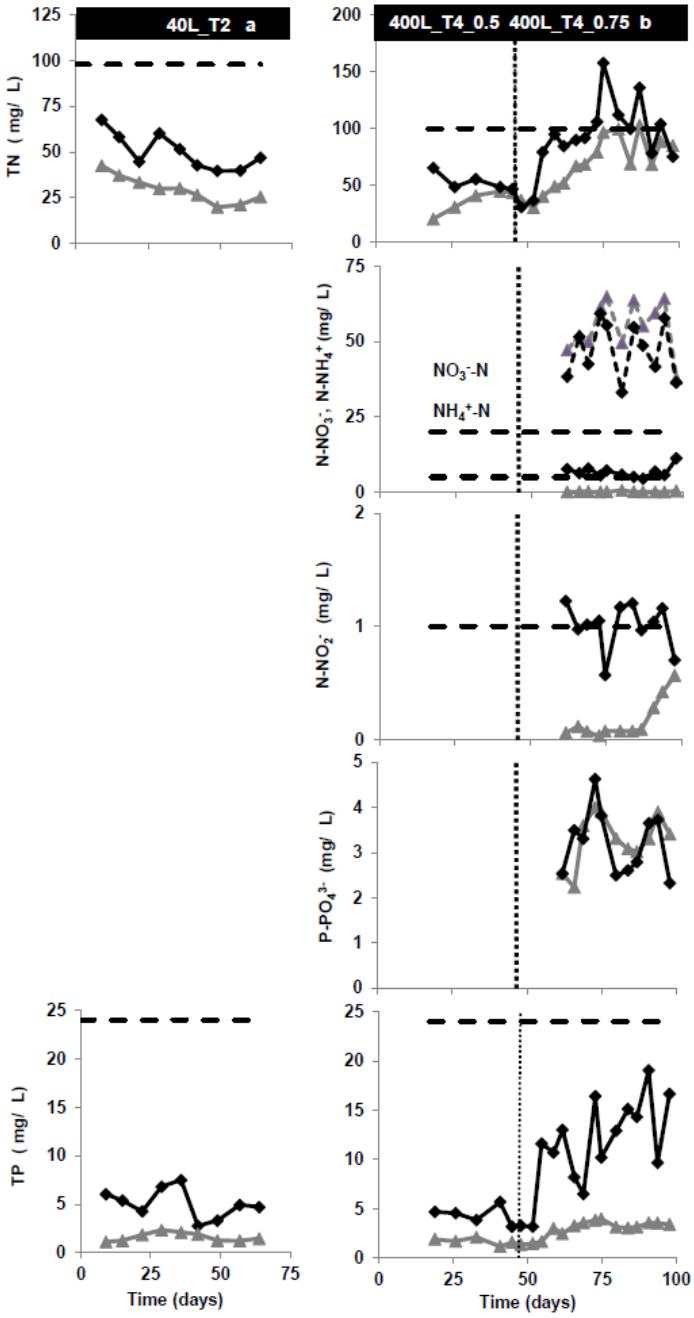
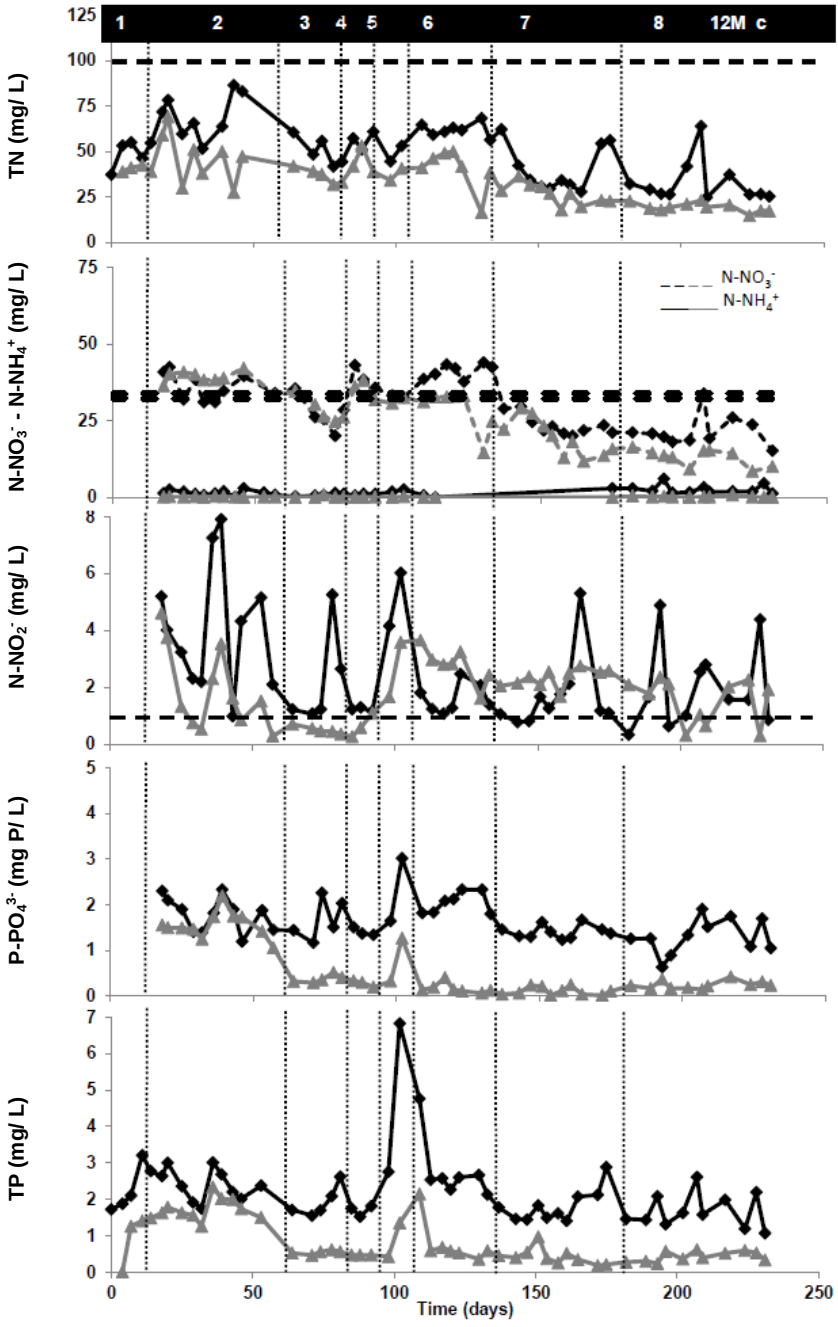


Fig. 7.4. *N and P in influent (♦) and effluent (▲) of aquaculture wastewater treating MaB-floc SBRs of 40 L (a), 400 L (b) and 12 m³ (c, on next page)*
Discharge norms (- - - - -) according to current (2013/09) environmental permit for this wastewater.



(Figure caption on previous page)

Table 7.2. *Removal rates of MaB-flocs SBRs treating aquaculture wastewater and scale-up conversion factors compared to 12M*

Reactor	**Removal rates (unit $L_{reactor}^{-1}$ day^{-1}) and scale-up conversion factors[c]**						
	EC ($\mu S\ cm^{-1}$)	**TIC (mg)**	**TOC (mg)**	**COD (mg)**	**BOD_5 (mg)**	**TN (mg)**	**TP (mg)**
4L_T2[a]	n.d.[b]	15.0 ± 7.0 (1.5)	14.0 ± 4.1 (4.7)	65 ± 6 (16)	19 ± 2 (18)	9.8 ± 1.8 (2.7)	1.56 ± 0.08 (5.4)
40L_T2	-41 ± 38 (-1.7)[c]	-7.5 ± 6.5 (-0.8)	19.5 ± 8.5 (6.6)	88 ± 26 (22)	49 ± 11 (46)	10.3 ± 2.7 (2.8)	1.73 ± 0.73 (6.0)
400L_T4_0.75	3 ± 4 (0.1)	2.6 ± 9.5 (0.3)	12.6 ± 7.5 (4.2)	38 ± 29 (9.5)	44 ± 20 (42)	4.2 ± 4.2 (1.2)	0.67 ± 0.29 (2.3)
400L_T4_0.50	9 ± 9 (0.4)	20.4 ± 13.6 (2.1)	21.8 ± 16.8 (7.4)	79 ± 29 (20)	44 ± 21 (42)	6.2 ± 4.5 (1.7)	2.10 ± 1.08 (7.2)
12M_02_T4_F0	5 ± 9 (0.2)	5.9 ± 5.4 (0.6)	5.6 ± 7.4 (1.9)	7 ± 4 (1.7)	3 ± 2 (2.9)	5.7 ± 4.0 (1.6)	0.18 ± 0.09 (0.6)
12M_03_T4_F0	-2 ± 7 (-0.1)	7.6 ± 2.2 (0.8)	3.1 ± 3.0 (1.1)	4 ± 7 (0.9)	0 ± 4 (0)	3.4 ± 1.2 (0.9)	0.35 ± 0.10 (1.2)
12M_04_T4_F3	15 ± 0 (0.6)	7.4 ± 3.1 (0.8)	3.5 ± 0.5 (1.2)	13 ± 13 (3.1)	1 ± 1 (0.6)	3.0 ± 3.1 (0.8)	0.31 ± 0.04 (1.1)
12M_05_T8_F3	16 ± 2 (0.7)	5.3 ± 0.1 (0.5)	0.8 ± 0.1 (0.3)	9 ± 16 (2.4)	1 ± 1 (0.7)	1.4 ± 0.2 (0.4)	0.49 ± 0.28 (1.7)
12M_06_T8_F0	25 ± 15 (1.0)	4.5 ± 1.0 (0.5)	2.3 ± 3.3 (0.8)	3 ± 4 (0.8)	0 ± 0 (0)	2.7 ± 1.8 (0.7)	0.25 ± 0.05 (0.9)
12M_07_T4_F0	45 ± 21 (1.9)	15.2 ± 3.6 (1.6)	0.9 ± 7.4 (0.3)	-2 ± 4 (-0.5)	0 ± 1 (0)	3.5 ± 3.2 (1.0)	0.35 ± 0.10 (1.2)
12M_08_T4_F5	41 ± 24 (1.7)	14.2 ± 2.7 (1.5)	3.1 ± 4.2 (1.0)	3 ± 5 (0.7)	1 ± 1 (0.8)	3.4 ± 2.5 (0.9)	0.31 ± 0.11 (1.1)
12M_all data[d]	24 ± 24 (1.0)	9.8 ± 5.8 (1.0)	3.0 ± 5.4 (1.0)	4 ± 7 (1.0)	1 ± 2 (1.0)	3.6 ± 3.0 (1.0)	0.29 ± 0.13 (1.0)

[a] Results of Chapter 6; [b] No data; [c] Values between brackets are scale-up conversion factors calculated as average removal rate for a certain reactor devided by the average removal rate of 12M_all ; [d] Average of all samples of period 2-8 of reactor 12M.

***Table 7.3**. Removal efficiencies of MaB-flocs SBRs treating aquaculture wastewater and scale-up conversion factors compared to 12M*

Reactor	Removal efficiencies (%) and scale-up conversion factors[c]						
	EC	**TIC**	**TOC**	**COD**	**BOD_5**	**TN**	**TP**
4L_T2[a]	n.d.[b]	36 ± 17 (0.8)[c]	71 ± 21 (2.1)	80 ± 7 (2.9)	87 ± 11 (1.6)	58 ± 11 (1.8)	89 ± 5 (1.4)
40L_T2	-15 ±13 (-1.0)	-15 ± 16 (-0.3)	63 ± 22 (1.9)	80 ± 10 (2.8)	99 ± 2 (1.9)	41 ± 8 (1.3)	65 ± 15 (1.0)
400L_T4_0.75	2 ± 2 (0.1)	7 ± 31 (0.2)	73 ± 31 (2.2)	68 ± 21 (2.4)	95 ± 11 (1.8)	29 ± 25 (0.9)	59 ± 13 (0.9)
400L_T4_0.50	4 ± 4 (0.3)	45 ± 16 (1.0)	77 ± 20 (2.3)	93 ± 6 (3.3)	98 ± 4 (1.8)	22 ± 20 (0.7)	70 ± 12 (1.1)
12M_02_T4_F0	3 ± 5 (0.2)	21 ± 20 (0.5)	43 ± 55 (1.3)	47 ± 23 (1.7)	100 ± 0 (1.9)	32 ± 18 (1.0)	28 ± 12 (0.4)
12M_03_T4_F0	-1 ± 4 (-0.1)	38 ± 7 (0.8)	42 ± 31 (1.3)	15 ± 35 (0.5)	83 ± 29 (1.6)	26 ± 5 (0.8)	71 ± 4 (1.1)
12M_04_T4_F3	8 ± 1 (0.5)	40 ± 14 (0.9)	57 ± 12 (1.7)	39 ± 41 (1.4)	50 ± 50 (0.9)	20 ± 21 (0.6)	72 ± 3 (1.1)
12M_05_T8_F3	19 ± 3 (1.2)	57 ± 2 (1.3)	29 ± 7 (0.9)	37 ± 76 (1.3)	100 ± 0 (1.9)	23 ± 0 (0.7)	82 ± 3 (1.3)
12M_06_T8_F0	27 ± 13 (1.7)	53 ± 7 (1.2)	30 ± 45 (0.9)	32 ± 48 (1.1)	19 ± 24 (0.4)	34 ± 20 (1.1)	74 ± 10 (1.2)
12M_07_T4_F0	25 ± 11 (1.6)	60 ± 13 (1.3)	-2 ± 74 (-0.1)	-25 ± 38 (-0.9)	3 ± 76 (0.1)	29 ± 22 (0.9)	75 ± 14 (1.2)
12M_08_T4_F5	25 ± 14 (1.6)	51 ± 6 (1.1)	51 ± 34 (1.5)	58 ± 57 (2.1)	57 ± 39 (1.1)	38 ± 12 (1.2)	73 ± 11 (1.1)
12M_all data[d]	16 ±15 (1.0)	45 ± 19 (1.0)	33 ± 51 (1.0)	28 ± 48 (1.0)	53 ± 56 (1.0)	31 ± 17 (1.0)	64 ± 22 (1.0)

[a] Results of Chapter 6; [b] No data; [c] Values between brackets are scale-up conversion factors calculated as average removal efficiency for a certain reactor divided by the average removal efficiency of 12M_all ; [d] Average of all samples of period 2-8 of reactor 12M

Table 7.4. *Biomass productivities and quantum yield of MaB-floc SBRs treating aquaculture wastewater, and scale-up conversion factors*

Reactor	Reactor performances					Scale-up conversion factors compared to 12M_all[b]				
	Volumetric biomass productivity ($mg\ L_{reactor}^{-1}\ day^{-1}$)		Production per $V_{influent}$ treated ($mg\ L_{influent}^{-1}$)		Max. Quantum Yield[a] (%)	Volumetric biomass productivity ratio		Production per $V_{influent}$ treated ratio		Max. Quantum Yield ratio
	TSS	VSS	TSS	VSS		TSS	VSS	TSS	VSS	
4L_T2[c]	236 ± 73	109 ± 30	472 ± 146	218 ± 60	6.5 ± 5.9	10.4	13.4	4.9	6.3	5.9
40L_T2	65 ± 8	45 ± 6	131 ± 16	91 ± 12	11.1 ± 10.1	2.9	5.5	1.4	2.6	10.1
400L_T4_0.75	14 ± 15	11 ± 12	55 ± 60	42 ± 49	4.0 ± 3.6	0.6	1.3	0.6	1.2	3.6
400L_T4_0.50	16 ± 23	12 ± 17	64 ± 92	48 ± 66	4.6 ± 4.2	0.7	1.5	0.7	1.4	4.2
12M_01_T8_F0	4 ± 6	3 ± 4	30 ± 47	25 ±35	1.3 ± 1.1	0.2	0.4	0.3	0.7	1.1
12M_02_T4_F0	7 ± 11	6 ± 9	29 ± 43	23 ± 36	1.3 ± 1.2	0.3	0.7	0.3	0.7	1.2
12M_03_T4_F0	21 ± 20	10 ± 11	83 ± 80	41 ± 44	1.2 ± 1.1	0.9	1.3	0.9	1.2	1.1
12M_04_T4_F3	34 ± 20	15 ± 8	136 ± 81	60 ± 32	1.4 ± 1.2	1.5	1.8	1.4	1.7	1.2
12M_05_T8_F3	17 ± 49	5 ± 40	134 ± 395	43 ± 318	0.6 ± 0.6	0.7	0.7	1.4	1.2	0.6
12M_06_T8_F0	6 ± 100	1 ± 31	45 ± 796	11 ± 246	0.6 ± 0.6	0.2	0.2	0.5	0.3	0.6
12M_07_T4_F0	47 ± 68	12 ± 19	176 ± 276	47 ± 77	1.2 ± 1.1	2.0	1.5	1.8	1.3	1.1
12M_08_T4_F5	33 ± 47	11± 12	132 ± 189	43 ± 50	1.0 ± 0.9	1.5	1.3	1.4	1.2	0.9
12M_all[d]	23 ± 54	8 ± 18	96 ± 337	35 ± 115	1.1 ± 1.0	1.0	1.0	1.0	1.0	1.0

[a] Highest quantum yield that is possible, meaning in case of no heterotrophic growth, no TSS or VSS addition by influent and no MaB-floc grazers; calculated as mol C produced per day on mol photons provided per day, based on 50 % C content of VSS; [b] Average value for a certain reactor divided by the average value of 12M_all; [c] Results of previous study (Chapter 6); [d] Average of all samples of period 1-8 of outdoor reactor 12M.

The molar ratios of removal rates are in line with the average TC:TN ratio and TP:TN ratio of microbial biomass (Geider and La Roche, 2002). Indeed, for each mol TN removed, 10.8 ± 11.4 mol TC was removed (averages of periods 1 and 2), and for each mol TP removed, 9.6 ± 8.4 mol TN was removed. However, per g VSS produced, 0.49 ± 0.42 g TN, 6.62 ± 3.80 g COD, 3.59 ± 2.90 g BOD_5, 1.08 ± 1.43 g TOC and 2.08 ± 2.56 g TC, 1.00 ± 1.64 g TIC and 0.15 ± 0.08 TP were removed (averages of period 1 and 2). This means that not all C and N removal could have been due to biomass growth. More research is needed to confirm which of the following removal mechanisms play a role in this: TN removal via denitrification, VSS removal by predators, TC removal via CO_2 emission to air and/or $CaCO_3$ precipitation.

During period 2, nitrogen species were studied more in detail (Fig. 7.4.b). On average, 97.8 ± 3.0 % of NH_4^+-N and 81.3 ± 23.0 % of NO_2^--N were removed. This resulted in effluent containing 0.15 ± 0.18 mg NH_4^+-N L^{-1} and 0.17 ± 0.18 mg NO_2^--N L^{-1}, and largely met the current discharge norms of 32 mg NH_4^+-N L^{-1} and 0.91 mg NO_2^--N L^{-1}. In contrast, the effluent contained 55.0 ± 8.60 mg N-NO_3^- L^{-1} and was largely above the current discharge norm of 33.9 mg N-NO_3^- L^{-1}. The N-NO_3^- concentration in the wastewater was on average with 18.2 ± 15.7 mg N-NO_3^- L^{-1} increased during treatment in the MaB-floc SBR (Fig. 7.4.b). The question remains whether this was due to nitrification, excretions by MaB-floc predators and/or lysis of organic compounds catalyzed by enzymes excreted by cyanobacteria, such as protease and urease (Thajuddin and Subramanian, 2005).

7.3.1.3. Outdoor MaB-floc SBR raceway pond of 12 m^3

Large diurnal fluctuations in the pH and DO concentrations of the raceway pond (referred to as 12M) were observed (Fig. 7.5.). These are typical for photosynthetic algae reactors (Richmond, 2004), but can lead to a reactor pH above the discharge norm of 9.5. Therefore, effluent was discharged after the pH decrease at night. During summer days with increased daily PPFD (Fig. 7.5.), the pH decrease during dark was not sufficient to reach the pH discharge norm of 9.5 (Fig. 7.3.c). Therefore flue gas sparging was needed. This was demonstrated by turning off the flue gas sparging during period 6 and 7. This resulted in an effluent pH of 9.78 ± 0.46 and 9.78 ± 0.51 in period 6 and 7, respectively (Fig. 7.3.c). Sparging 5 % CO_2, representing flue gas of natural gas burning (Chapter 4), at 0.00039 vvm during period

8 in 12M led to a dischargeable effluent pH of 9.08 ± 0.22. Compared to previous studies with flue gas sparged MaB-floc reactors (Chapter 3), low volumetric flue gas loading rates (FGLR) were applied. This is of importance because a decreased FGLR means (1) less gas pumping and thus lower energy and maintenance costs, and (2) a smaller number of flue gas blower systems in the raceway pond and thus decreased capital and maintenance costs. Moreover, by applying a low FGLR, an increase in TIC in the effluent was avoided (Fig. 7.3.c). The RR of TIC doubled in time (Table 7.2.). The main TIC removal mechanism was not the biomass productivity, because these parameters were not significantly correlated. The TIC RR only poorly correlated with the daily PPFD ($r_{sp} = 0.296$, $p < 0.05$). Photo-saturation and photo-inhibition at high PPFDs (Richmond, 2004), varying TIC production in the reactor and/or TIC removal via $CaCO_3$ precipitation could explain this. Similar to the TIC RR, the EC RR increased in time (Table 7.2.). The TIC RR was positively correlated with the EC RR ($r_s = 0.454$, $p < 0.005$), the ash content of MaB-flocs ($r_s = 0.564$, $p < 0.001$) and the $T_{reactor}$ ($r_s = 0.624$, $p < 0.001$). These observations support our hypothesis that the increased TIC removal was mainly due to carbon scavenging by $CaCO_3$ precipitation. This $CaCO_3$ precipitation increases by increasing temperature and decreases the EC by removing calcium.

The COD and BOD_5 of the effluent reached the current discharge norms, but their influent concentrations were in most cases already below the discharge norms (Fig. 7.2.c). The influent COD (56 ± 35 mg L^{-1}) showed strong variations in time (Fig. 7.2.c). This led to low and strongly varying REs (Table 7.2.). The loading rates of 12 ± 7 mg COD $L_{reactor}$ day^{-1} and 2 ± 2 mg BOD_5 $L_{reactor}$ day^{-1} were very low. This resulted in DO concentrations in reactor 12M above 5 mg L^{-1} during most of the operation period, even at night, except during period 8 (Fig. 7.5.). The supersaturated DO values up to 24 mg L^{-1} (Fig. 7.5.) demonstrate photosynthetic aeration by MaB-flocs and BOD5 underloading of 12M. These supersatured DO values can be toxic for microalgae (Richmond, 2004). Therefore, during further optimisation research should investigate the potential of increased BOD_5 loadings on the reactor performance, for example by altering the influent pretreatment step.

The influent TN concentration of reactor 12M decreased in time from 68.4 ± 12.4 g L^{-1} in period 2 to 32.7 ± 11.7 g L^{-1} in period 8; all of which are under the discharge norm (Fig. 7.4.c). On average, only around 50 % of TN was removed in

reactor 12M (Table 7.2.). The TN RR was not significantly correlated with the daily PPFD or any other nutrient removal rates. All influent and effluent concentrations of $N\text{-}NH_4^+$ were below the discharge norm (Fig. 7.4.c). The NH_4^+ RE varied during period 2-3, but was more stable during period 4-8 (Fig. 7.4.c). The NO_3^- in the influent varied in time and reached values above the discharge norm (Fig. 7.4.c). The NO_3^- RE was negative during the start-up in period 2, but increased in time from period 3 to 8 (Fig. 7.4.c). The current discharge norms for NO_3^- were met, except during period 2, 3 and 4. Since the NO_3^- RR was positively correlated with the PPFD ($r_s = 0.499$, $p < 0.001$), $T_{reactor}$ ($r_s = 0.429$, $p < 0.005$) and the RR PO_4^{3-} ($r_s = 0.429$, $p < 0.005$), the weather conditions and/or a phosphorous limitation could be a reason for the decreased NO_3^- RR.

The influent contained on average 2.50 ± 1.83 mg $N\text{-}NO_2^-$ L^{-1} which is largely above the discharge norm (Fig. 7.4.c). Only during period 3, the effluent met the discharge norm for $N\text{-}NO_2^-$ (Fig. 7.4.c). The NO_2^- RE decreased in time (Fig. 7.4.c). Negative NO_2^- REs were obtained despite the aerobic conditions needed for nitrification. Further optimisation to lower the NO_2^- concentrations is crucial. Since the NO_2^- concentration in the indoor fish tanks was lower than 1 mg L^{-1}, the NO_2^- must have increased during storage in the influent buffer tanks. Therefore a possible strategy to enhance the NO_2^- of the effluent would be to decrease the HRT of the influent buffer tanks by discontinuous feeding of the MaB-floc SBR during the day.

Very low average effluent values of 0.80 ± 0.60 mg TP and 0.54 ± 0.60 mg $P\text{-}PO_4^{3-}$ L^{-1} were obtained; both below their discharge norm (Fig. 7.4.c). These values decreased in time, reaching a minimum during period 7 (0.42 ± 0.23 mg TP L^{-1} and 0.11 ± 0.10 mg $P\text{-}PO_4^{3-}$ L^{-1}). The PO_4^{3-} RR was positive correlated with the daily PPFD ($r_s = 0.486$, $p < 0.001$) and $T_{reactor}$ ($r_s = 0.462$, $p < 0.005$). For each mol TP removed, 26.1 ± 20.5 mol TN was removed. These results together with the high TN:TP ratio of the effluent (128 ± 60) suggest that P limited the N removal via biomass growth. The above results highlight the large potential of MaB-floc SBRs for phosphorous polishing and recovery, both of large interest nowadays (Shilton et al., 2012).

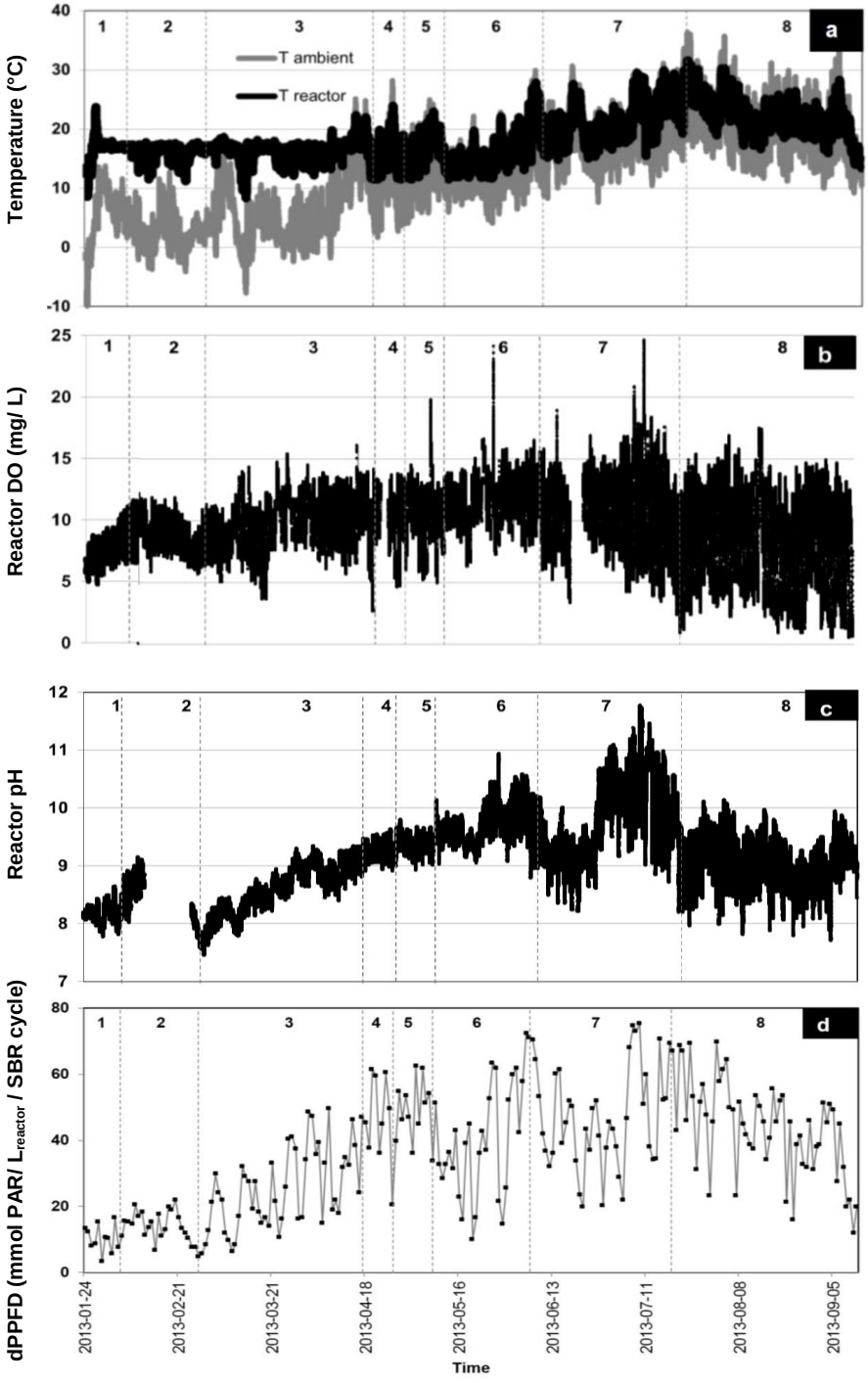


Fig. 7.5. *Ambient and reactor T (a), reactor DO (b), reactor pH (c) and dPPFD (d) of the 12 m³ outdoor MaB-floc raceway pond*

7.3.1.4. Effect of up-scaling on wastewater treatment

In spite of the varying wastewater composition, biomass density and HRT, some general conclusions can be made on the effect of up-scaling of MaB-floc SBRs on wastewater treatment.

Firstly, up-scaling to outdoor conditions strongly increased the effluent pH. Outdoors, flue gas sparging was needed to obtain an effluent pH below the discharge norm. Despite being advantageous in terms of greenhouse gas mitigation, flue gas sparging in wastewater treatment raceways represents an extra cost (up to 20 % of the capital costs; Zamalloa et al., 2011). In certain countries, such as Belgium, this cost cannot be compensated by revenues for CO_2 credits because no such credits can be obtained for flue gas scrubbing in open ponds.

Secondly, up-scaling not always decreased, but for some nutrients increased the nutrient removal (Table 7.2.; Table 7.3.). Indeed, the TIC RR in reactor 12M was in the same order as in the 4 L and 400 L reactor, but was higher than the negative values obtained in the 40 L reactor. In winter, the EC RR of reactor 12M was similar as these values of the 400 L reactor, but in summer these values increased with a factor 6. The same trend was observed for the TIC RE. Up-scaling to outdoor raceway pond decreased the RR of TOC, COD and BOD_5 with a factor up to 46. This was mainly due to their decreased concentrations in the influent during up-scaling. In contrast, the REs of these parameters were decreased with a factor 2-3 while up-scaling. The TN RR outdoors were decreased with a factor 3 compared to the 4 L and 40 L reactors, but was similar to the 400 L reactor. The TN RE in 12 M decreased only with a factor 1-2 compared to the 4 and 40 L reactor. The phosphorous removal followed the same trend. In general, the conversion factors were less varying for the RE compared to those for the RR. This means that extrapolation of wastewater treatment reactor performances from lab to outdoor pilot scale, as a first rough assessment, should be based on RE rather than on RR. Overall, the nutrient REs from the wastewater were decreased with a factor 1-3 during up-scaling.

Thirdly, up-scaling increased the HRT to 4 days. This means that per m^3 indoor fish tank, a microalgae raceway pond area of 1 m^2 would be needed (daily discharge of 10 % of the fish tank water). To overcome potential problems of regarding land availability, raceway ponds on the roof of the indoor aquaculture

facility could be a worthwhile option to investigate. If year round wastewater treatment is targeted, pond heating with waste heat will be needed.

7.3.2. MaB-floc characteristics

A first important requirement of MaB-flocs is adequate settling, because it is crucial for safeguarding the discharge of biomass-free effluent and high biomass recovery. This means that at the end of the dark period of the SBR operation, the volume of the settled MaB-flocs should be lower than the effective reactor volume after effluent withdrawal. In all MaB-floc SBRs, this was obtained. Indeed, the fact that maximum 50 % of the effective reactor volume was withdrawn during effluent discharge, that the average dSVI of all SBRs was lower than 250 mL g^{-1} TSS (Fig. 7.6.a) and that the TSS values were lower than 2 g TSS L^{-1} (Fig. 7.6.e; Fig. 7.6.f) confirms this. The dSVI significantly decreased while up-scaling from 4 L over 40 L to 400 L, but significantly increased again in 400L_T4_0.50 (Fig. 7.6.a). This was related to the dominance of filamentous cyanobacteria. In 12M, the dSVI (Fig. 7.6.a) and settled MaB-floc density (Fig. 7.6.b) remarkably decreased in time. This dSVI showed a strong positive correlation with the VSS:TSS ratio of the MaB-flocs (r_s = 0.935; $p < 0.001$) (Fig. 7.6.g). The VSS:TSS ratio decreased in time from 79.1 ± 1.5 % in 12M_02_T2_F0 to 30.7 ± 3.1 % in 12M_08_T2_F5 (Fig. 7.6.c).

Microscopic observations showed that a large amount of crystals were present in the MaB-flocs (Fig. 7.7.). Up to 30 % of the ash of MaB-flocs was calcium (Chapter 8). This suggests that these crystals were $CaCO_3$ (around 70 % of ash). XRD-analyses are needed to confirm if it was calcite. Next to MaB-floc settling, an increased $CaCO_3$ content of MaB-flocs is of interest for carbon capture and removal of wastewater hardness. However, for biomass valorisation as aquaculture feed ingredient or feedstock for biogas production, it might be disadvantageous due to the decreased energy content of the biomass, imbalanced Ca:P:K ratio of the biomass and reactor scaling.

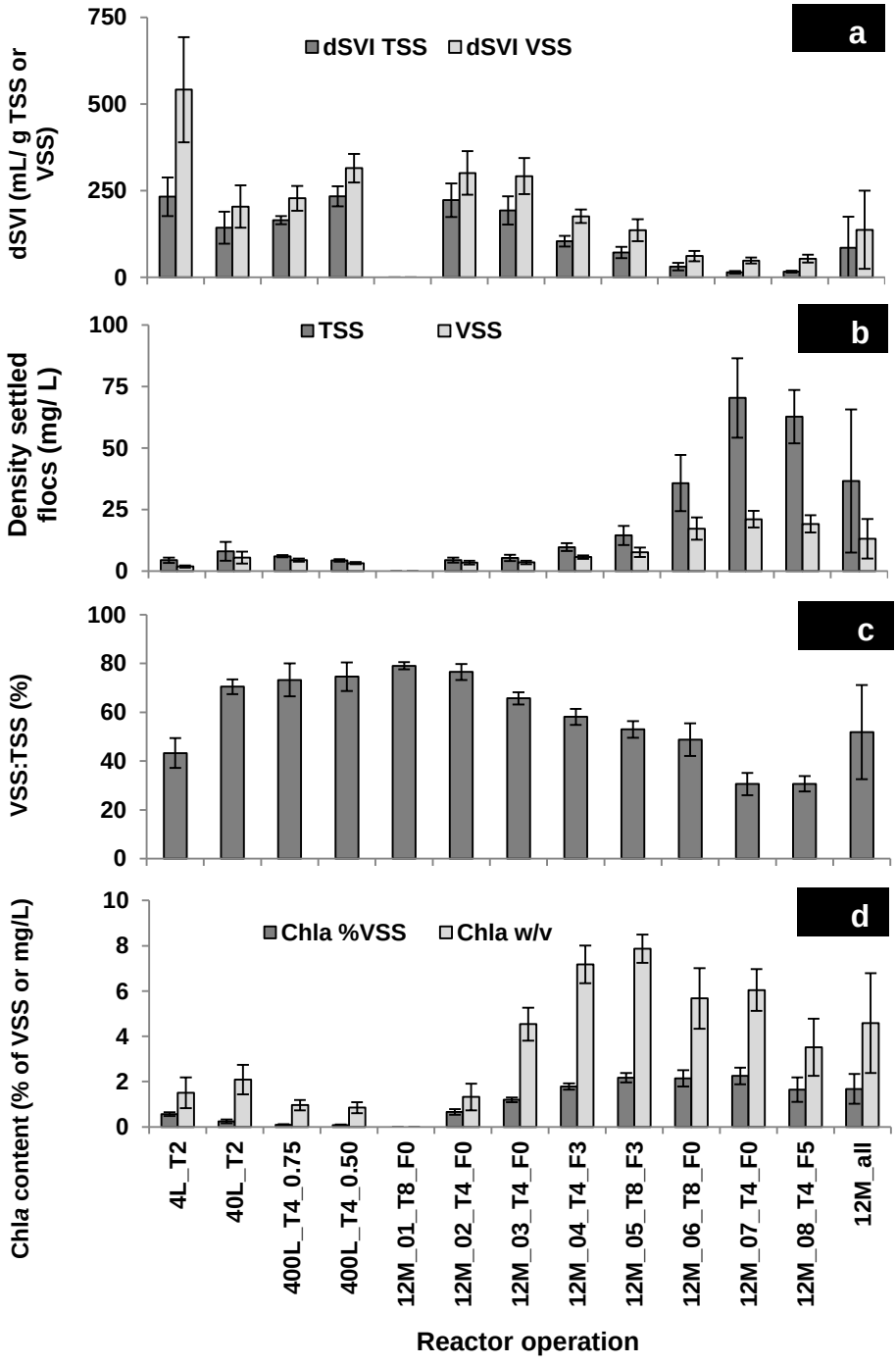
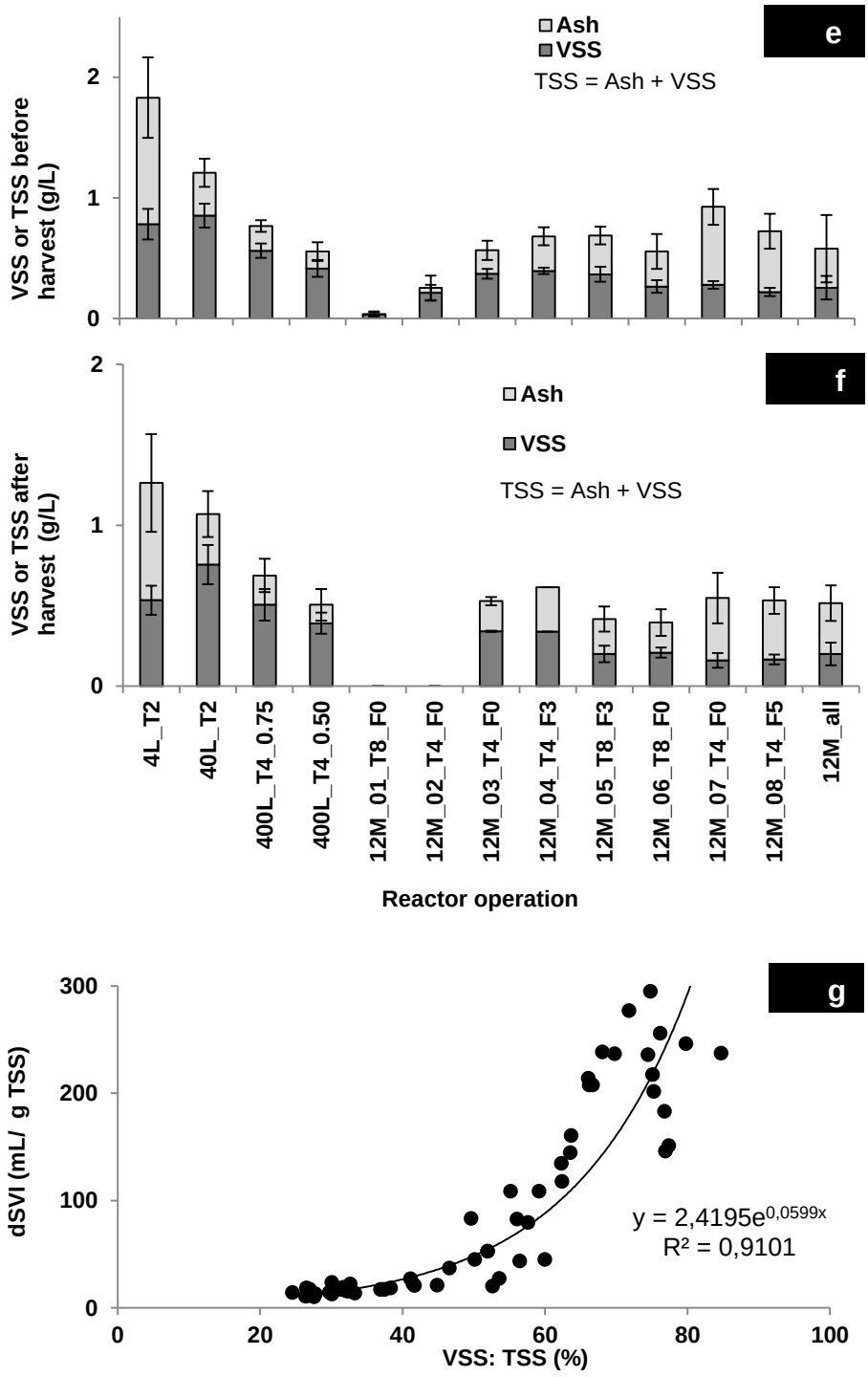


Fig. 7.6. *MaB-floc characteristics of SBRs of 4 L, 40 L, 400 L and 12 m^3: dSVI (a), density of settled MaB-flocs (b), VSS:TSS (c), chlorophyll a content (d), density before harvesting (e), density after harvesting (f) and correlation between VSS:TSS and dSVI (g)* *(Figures f-g on next page)*



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For biomass dewatering and valorisation, the size, structure and abundance of the most dominant photosynthetic microorganisms (PM) present in MaB-flocs is of importance. During up-scaling, the dominant PM in MaB-flocs changed from filamentous cyanobacteria (*Phormidium* sp.) indoors to filamentous microalgae (*Ulothrix* sp. and/or *Klebsormidium* sp.) in reactor 12M (microscopic observations; Fig. 7.7.). Filamentous PM are suitable candidates for wastewater treatment, because they can be harvested relatively easily (Markou and Georgakis, 2011). To the best of our knowledge, this is the first report on dominance of this microalgal species in an outdoor raceway. Although *Ulothrix* sp. are not commercially cultured, these species can be interesting in aquaculture to increase fish fertility and as source of unsaturated fatty acids 16:4ω-3 and 18:3ω-3 (Jameison and Reid, 1976). Jaya Prakash Goud et al. (2007) observed antibacterial activity of extracts of *Ulothrix* sp. towards *Bacillus cerius*, *Bacillus subtilis*, *Salmonella typhimurium* and *Escherichia coli*. Moreover, these authors found that this specie contained the highest amount of chlorophyll *a* (5.6 mg g^{-1}) and carotenoids (4.5 mg g^{-1}) of all 24 studied freshwater algal species.

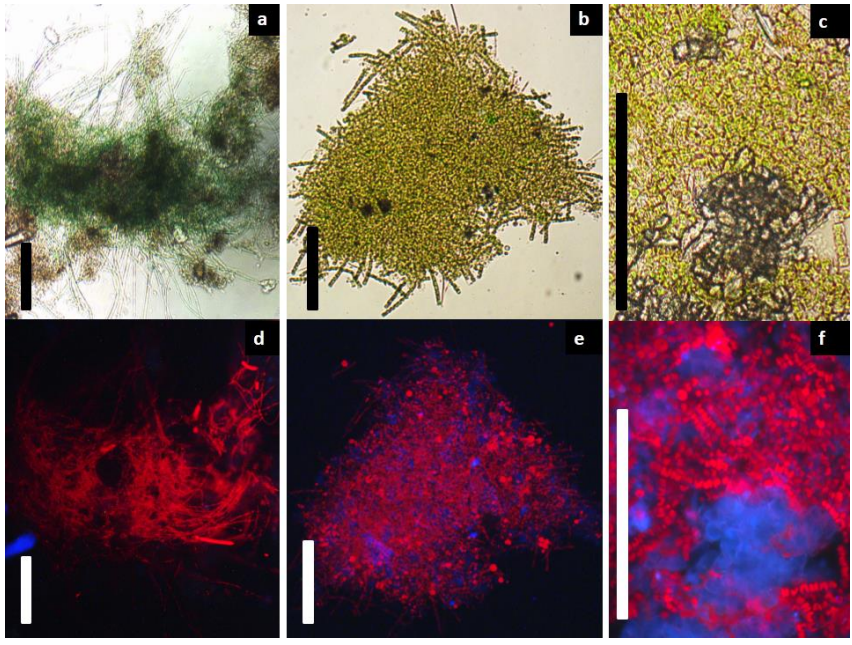

Fig. 7.7. *Light microscopy (top row) and fluorescence microscopy images (bottom row) of MaB-flocs from 400L_T4_0.50 (a, d) and outdoor 12M (b, c, e, f)*
On fluorescence microscopy images, chlorophyll appears in red and crystals in blue. Depicted scale bar measures 100 µm in length.

During spring, the growth of *Scenedesmus dimorphus* in the outdoor influent tank led to the unwanted presence of this non-flocculating species in the effluent. Covering the influent tank, solved this problem. This demonstrates that SBR operation can avoid the dominance of non-flocculating microalgae in a MaB-floc raceway pond, even with a high HRT of 8 days.

Up-scaling to 400 L significantly decreased the Chl*a* content of the MaB-flocs (Fig. 7.6.d). This was probably due a combination of the decreased PPFD (Table S1) and increased BOD_5 loading (Fig. 7.2.a; Fig. 7.2.b), favoring the growth of bacteria. In contrast, in reactor 12M, the Chl*a* content of MaB-flocs increased from 0.66 ± 0.13 % of VSS in 12M_02_T4_F0 to 1.64 ± 0.54 % of VSS in 12M_08_T4_F5 (Fig. 7.6.d). These values are in the upper range of those of pure microalgae or cyanobacteria (0.17 to 4.36 % of TS; Piorreck et al., 1984) and higher than that of earlier reports for *Ulothrix* sp. (0.56 % of TS; Jaya Prakash Goud et al., 2007) and *Phormidium* sp. (0.01-0.16 % of TS) (Bhattacharya and Pal, 2012). This indicates a high microalgae content in MaB-flocs, a hypothesis which was confirmed by microscopy (Fig. 7.7.). The Chl*a* content of MaB-floc VSS was negatively correlated with the dSVI (r_s = -0.750; $p < 0.001$), and positively correlated with the ash content (r_s = 0.632; $p < 0.001$) and daily PPFD (r_s = 0.534; $p < 0.001$), and not significantly correlated with the $T_{reactor}$. A possible explanation could be that the increased crystal content of MaB-flocs led to an increased light reflection, leading to an increasing Chla content of the microalgae. The average $A664_b$:$A665_a$ ratio was above 1.63 in all reactor set-ups. This demonstrates an adequate physiological condition of the PM in all SBRs (Van Den Hende et al, 2011a). It indicates that nor the increased ash content of MaB-flocs nor sparging of flue gas containing NO and SO_2, which can be toxic to some algal species (Chapter 4), were severely toxic for the MaB-flocs.

To conclude, up-scaling from indoor reactors to an outdoor raceway pond led to a drastic shift in the community structure, increased the ash and Chl*a* content of the MaB-flocs and enhanced the floc settling. These changes highlight the importance of outdoor pilot scale studies especially with respect to floc settling and biomass valorisation perspectives.

7.3.3. MaB-floc productivity

The average MaB-floc productivity was low (Table 7.4.), resulting in 9.2 g TSS and 3.2 g VSS per m^{-2} pond area day^{-1}, or 33 ton TSS and 12 ton VSS ha^{-1} pond area $year^{-1}$. Compared with indoor experiments, these values are 10-13 times lower, except for reactor 400L (Table 7.4.). They are 1.5-4 times lower compared to the result of 13-35 g TS m^{-2} pond area day^{-1} obtained by Park et al. (2011b) for wastewater treating algae raceway ponds. One of the reasons for this was the temporarily increased HRT. Indeed, during periods 5 and 6 of 12M with both a HRT of 8 days, the biomass productivity was only 70 and 20 % of the average productivity in 12M, respectively. In summer (period 7 and 8), 16 g TSS and 5 g VSS m^{-2} day^{-1} were produced. This is still lower than 25 g m^{-2} day^{-1} obtained during summer by Park and Cragss (2010) in a wastewater treating algae pond. Another reason was biomass loss due to predators. The fact that 25 % of the measured biomass productivities (68 in total) were negative and MaB-floc predators were observed in 12M (mainly larvae of *Tubifex* sp.; around 10-20 per kg dewatered MaB-flocs), confirms this. The presence of *Tubifex* sp. in the biomass can be beneficial for valorisation as aquaculture feed ingredient, since *Tubifex* sp. can increase appetites and palatability of fish (Lietz, 1988).

The MaB-floc productivities showed no significant positive correlation with the RRs of C, N and P parameters. This discrepancy could be due to biomass loss by predators, as observed in previous studies (Weinberger et al., 2012; Mulbry et al., 2008) and/or nutrient removal via other mechanisms such as denitrification, precipitation and adsorption (Shilton et al., 2012). To be realistic, until a sustainable and lucrative pathway of biomass valorisation is demonstrated, an increased biomass production per m^3 wastewater is actually not wanted, since this would only represent extra costs for MaB-floc harvesting and dewatering.

Table 7.5. *Biomass loss during harvesting by settling, gravity filtering and press filtering of outdoor MaB-flocs, and TS and VS content of the resulting dewatered MaB-floc cake*

Reactor operation (amount of harvests)	**MaB-floc loss (%)**							
	Settling		**Gravity filtering**		**Press filtering**		**Total**	
	TSS	**VSS**	**TSS**	**VSS**	**TSS**	**VSS**	**TSS**	**VSS**
12M_04_T4_F3 (1)	4.7	6.3	1.1	1.0	0.00	0.00	5.8	7.4
12M_05_T8_F3 (2)	13.9 ± 0.3	14.2 ± 8.8	1.9 ± 0.4	2.2 ± 1.0	0.08 ± 0.02	0.08± 0.03	15.9 ± 0.6	16.5 ± 9.9
12M_06_T8_F0 (3)	15.6 ± 3.2	20.8 ± 4.1	2.7 ± 0.8	3.1 ± 0.9	0.07 ± 0.01	0.12 ± 0.02	18.1 ± 2.4	24.0 ± 3.2
12M_07_T4_F0 (6)	8.8 ± 4.0	20.2 ± 11.3	1.4 ± 0.7	2.8 ± 1.7	0.05 ± 0.03	0.12 ± 0.06	10.2 ± 4.2	23.1 ± 12.1
12M_08_T4_F5 (8)	3.3 ± 3.4	6.9 ± 6.1	0.4 ± 0.3	0.9 ± 0.8	0.04 ± 0.04	0.10 ± 0.08	3.7 ± 3.7	7.8 ± 6.8
12M_All (20)	7.9 ± 5.7	13.6 ± 9.8	1.2 ± 0.9	1.9 ± 1.4	0.05 ± 0.03	0.10 ± 0.07	9.1 ± 6.4	15.7 ± 10.9

Reactor operation (amount of harvests)	**Solid content of MaB-floc cake**	
	(%TS)	**(%VS)**
12M_04_T4_F3 (1)	24.6	no data
12M_05_T8_F3 (2)	34.2 ± 2.8	24.8 ± 0.9
12M_06_T8_F0 (3)	33.7 ± 6.8	23.6 ± 3.0
12M_07_T4_F0 (6)	49.7 ± 5.5	25.6 ± 3.8
12M_08_T4_F5 (8)	46.6 ± 3.1	25.7 ±4.8
12M_All (20)	42.9 ± 8.7	25.3 ± 3.8

The maximum quantum yields (Y_{qmax}) of each reactor were calculated as daily mol C-VSS produced per daily mol PAR photons provided, assuming that the by microalgae dominated VSS contained 50 % C (Van Den Hende et al., 2012a). This Y_{qmax} is not the actual but a maximum possible value, because VSS could also have been produced heterotrophically or added with influent feeding. Since in photosynthesis, 8 mol photons are needed to convert 1 mol inorganic C to organic C, and around 10-20 % of the light is lost due to reflection on the pond surface (Park et al., 2011b), maximum Y_{qmax} values of 8-9 % are expected in case of no photo-saturation like in indoor reactors. The high values obtained in 40L_T2 (Table 7.3.) demonstrate that VSS productivity in this reactor was not only due to photosynthetic growth. Since photosynthesis of most algal species is saturated at a PPFD of around 200 µmol PAR photons m^{-2} s^{-1}, which is about 10-17 % of summer/winter maximum outdoor PPFD (Park et al., 2011b), more realistic Y_{qmax} values for outdoor reactors range between 0.8 % and 1.5 %. The Y_{qmax} obtained for 12M (Table 7.3.) was 1.1 ± 1.0 % and thus in this range. The 60 % lower Yqmax values obtained during period 5 and 6 (Table 7.3.) with a doubled HRT indicate that light was not the reason for the decreased biomass productivity during these periods. Overall, scale-up lowered the Y_{qmax} values to realistic values for outdoor photosynthetic growth.

7.3.4. MaB-floc harvesting

An efficient and cost-effective biomass harvesting is a key factor for a sustainable wastewater treatment by microalgae (Udom et al. 2013; Park et al., 2011). In this study, harvesting of MaB-flocs proceeded in two steps: (1) concentration by gravity settling in a settling tank, and (2) dewatering of the settled biomass by filtering. For cost-effective harvesting of MaB-flocs on an industrial scale, it is important that floc settling is fast and that it leads to high enough MaB-floc densities, to reduce the settling tank dimensions and pumping. Both on lab and on pilot scale, MaB-flocs settled fast. On lab-scale, the settled MaB-floc densities ranged between 4 and 8 g TSS L^{-1} and 2 and 6 g VSS L^{-1} (Fig. 7.6.b). In 12M, these values increased from 4.5 ±1.0 g TSS L^{-1} and 3.5 ± 0.8 g VSS L^{-1} in period 2 to the 70.3 ± 16.2 g TSS L^{-1} and 21.1 ± 3.3 g VSS L^{-1} in period 7 (Fig. 7.6.b). Gravity settling during harvesting in reactor 12M increased the MaB-floc TSS densities with a factor up to 90. Settling was the harvesting step with the highest MaB-floc loss (Table 7.5.). The MaB-floc losses

were still in the same range as compared to microalgae settling by flocculant addition (2-15 % TSS, after 1 h settling) (Udom et al., 2013) and by bioflocculation in a biomass recycling raceway (14-75 % TSS, yearly average after 1 h settling) (Park et al., 2011a). In our study, the remaining biomass in the supernatant was actually not lost, because it was pumped back into the pond to enable pond stirring without decreasing the HRT (i.e. adding extra influent) and to increase biomass recovery during the 10 times longer settling period at dark.

During the second harvesting step, the settled MaB-flocs were pumped in a filter bag and dewatered by gravity filtering followed by hydropress filtering. A relatively large pore size of 150-250 µm was used (Uduman et al., 2010). Dewatering increased the TSS densities of settled MaB-flocs with a factor up to 27. Dewatering led to a MaB-floc cake of 25-50 %TS (Table 7.5.). This is higher compared to the resulting 21 %TS in the previous lab-scale study (Chapter 6), and in the mid-range compared to 3-90 %TS obtained for other algae dewatering systems (Udom et al., 2013; Uduman et al., 2010). During dewatering, the loss of VSS was always higher than of TSS (Table 7.5.). As for gravity filtering, this was due to the loss of microalgae passing through the filter pores. As for press filtering, no microalgae were observed in the filtrate but the orange filtrate color suggests leakage of cell content due to the applied high pressure of 4 bar. On industrial scale, a lower pressure of the filter press will be applied. The gravity filtrate and press filtrate had a volume of 18 % and 1.5 % of total harvested reactor liquor. The filtrate contained 0.044 ± 0.025 and 0.046 ± 0.044 g TSS L^{-1}, and 0.029 ± 0.017 and 0.033 ±0.031 VSS g L^{-1}, respectively. This resulted in a discharged filtrate containing 0.044 ± 0.023 g TSS L^{-1} and 0.029 ± 0.016 g VSS L^{-1}. This is largely under the current discharge norm for this pikeperch culture wastewater of 1000 g suspended solids (SS) L^{-1}.

In this study, a hydropress was tested which uses water pressure of 4 bar and no electricity. Per kg dry biomass, 40 L of water was needed to dewater 1 kg of biomass. This has a cost of around 4 € m^{-3} (if drinking water is used and based on the prices in Belgium) or 0.16 € kg^{-1} dry MaB-flocs. On industrial scale, an electricity-powered belt filter press will be a cheaper option, having an operational cost of 0.4-0.7 kWh kg^{-1} dry matter (Udom et al., 2013) and resulting in 0.04-0.07 € kg^{-1} dry matter. To conclude, despite the drastic change of dominant microalgal species in the MaB-flocs, a floc recovery of 98.8 ± 0.9 % of TSS and 98.0 ± 1.5 % of VSS showed

similar results compared to lab-scale. MaB-floc dewatering showed better results compared to lab-scale. These promising results warrant further up-scaling with industrial belt filter presses.

7.4. Conclusions

MaB-floc SBRs treating aquaculture wastewater were up-scaled from indoor reactors to a 12 m^3 outdoor raceway pond. This scale-up decreased the nutrient removal efficiency and biomass productivity with a factor 1-13. Current discharge norms were reached, except for nitrate and nitrite. Outdoors, flue gas sparging was needed to lower the effluent pH. Both settling and ash content of MaB-flocs strongly increased during summer. Bioflocculation enabled successful harvesting by gravity settling and dewatering by filtering at 150-250 µm. Future research should focus on nitrogen removal and biomass valorisation, e.g., as ingredient for aquaculture feed.

7.5. Acknowledgements

This work was supported by the INTERREG IVB NWE Programme, the Flemish Government, Province West-Flanders, Howest and Ghent University LIWET within the EnAlgae project. The authors greatly acknowledge Bebouwen en Bewaren nv and CATAEL bvba for pilot reactor construction and discussion of results; Inagro for location, wastewater, electricity and discussion of results. P. Mahieu (Howest), G. Bourgeois, L. Buyse and S. Teerlinck (Inagro) for their technical assistance; J. Van Wichelen (UGent) for microalgae identification; E. Cocaud, M. Begué and C. Laurent (Howest students) for help in harvesting; R. Beyaert for making all filter bags; E. Vuylsteke and K. Debevere (UGent, EnAlgae) for proof-reading the manuscript.

Every element in an eco-design should provide at least 3 functions.

(Mollison, 1988)

Pictures on previous page:

Top: Bottles with pikeperch culture wastewater, MaB-flocs, effluent, dewatered MaB-flocs, dried MaB-flocs, shrimp feed containing MaB-flocs, uncooked and cooked shrimp *L.vannamei* (Boone, 1931) (from left to right).

Middle: Shrimp feed without and with MaB-flocs, dried MaB-flocs, uncooked shrimp and a cooked shrimp *L.vannamei* (Boone, 1931) (from top to bottom).

Bottom: Dried MaB-flocs.

CHAPTER 8

Microalgal bacterial flocs originating from aquaculture wastewater treatment as diet ingredient for Pacific white shrimp

Abstract

Microalgal bacterial flocs (MaB-flocs) in sequencing batch reactors are a novel and promising technology to treat aquaculture wastewater. To improve the economics of this technology, the harvested MaB-flocs should be valorised. Therefore, we investigated if MaB-flocs from an outdoor pilot reactor treating pikeperch (*Sander lucioperca* L.) wastewater can be used as an ingredient for shrimp diets. Considering the nutritional composition and high ash content of MaB-flocs, five shrimp diets were formulated by replacing mainly wheat (0-49 %) with MaB-flocs (0-8 % of the total diet). These diets were continuously fed to juvenile Pacific white shrimps (*Litopenaeus vannamei* Boone, 1931) cultured in a hybrid recirculating aquaculture system. The diet modifications did not affect the shrimp survival, weight gain, size distribution and food conversion rate, nor did they affect the proximate composition and fatty acid profile of the raw shrimp muscle. However, increasing the amount of MaB-flocs in shrimp diets significantly increased the pigmentation (redness and yellowness) of cooked shrimp tails. This shows that MaB-flocs originating from treatment of pikeperch wastewater can substitute 8 % of the diet ingredients of Pacific white shrimp while enhancing its pigmentation.

Chapter redrafted after:

Van Den Hende, S., Claessens, L., De Muylder, E., Boon, N., Vervaeren, H. Microalgal bacterial flocs originating from aquaculture wastewater treatment as diet ingredient for Pacific white shrimp. Submitted.

8.1. Introduction

As the Western aquaculture industry develops more intensively, its environmental impact increases (Crab et al., 2007). Compliance with water discharge regulations is likely to be one of the most limiting factors facing the further expansion of aquaculture (Martins et al., 2010). Therefore, cost-effective wastewater treatment systems including nutrient recovery and reuse of byproducts are of great interest to aquaculture worldwide. A sequencing batch reactor with microalgal bacterial flocs (MaB-floc SBR) is a novel sunlight-based system to treat wastewater via photosynthetic aeration without the need for mechanical aeration (Van Den Hende et al., 2011a; 2011b). During this aerobic treatment process, wastewater nutrients are recovered into microalgal bacterial biomass. MaB-flocs consist of a consortium of microalgae, cyanobacteria and bacteria and may include small amounts of rotifers, ciliates and precipitates (Van Den Hende et al., 2011b). The surplus of this biomass should to be harvested to keep the MaB-flocs in their exponential growth phase. In comparison to other microalgal systems, MaB-flocs settle by gravity and can be dewatered by a filter press with a relatively large pore size (Chapter 7). To further improve the economics of this MaB-floc system and to aim for an integrated aquaculture system, this MaB-floc biomass originating from treatment of aquaculture should be valorised into, for example, feed for aquaculture.

In case MaB-flocs are used to treat wastewater from culture pikeperch, the reuse of these microalgae containing flocs as feed ingredient for pikeperch is not recommended because of biosecurity/health reasons. This is because pikeperch is a carnivorous fish and in ponds reared on a diet of zooplankton, zoobenthos and fodder species (Craig, 2000). In contrast, penaeid shrimp are omnivores and ingest phytoplankton in their natural habitat (Senanan et al., 2009). Moreover, consumption of periphyton or bioflocs containing microalgae has shown to significantly improve the growth of penaeid shrimp, as for instance *Penaeus esculentus* (Burford et al., 2004), *Litopenaeus vannamei* (Audelo-Naranjo et al., 2011) and *Penaeus monodon* (Anand et al., 2013). Besides, shrimp farming is one of the fastest growing aquaculture sectors in the world and shrimp production dominates the aquaculture production by value (FAO, 2010). On the other hand, up to 50-60 % of the operational costs of intensive shrimp farming are the commercial diets (Wasielesky et al., 2006), while prices of raw materials for shrimp culture are likely to continue to increase (Naylor et

al., 2009). Therefore, the partial replacement of shrimp feed ingredients with wastewater by-products such as MaB-flocs could represent a valuable alternative especially if it leads to an increased growth and/or enhanced shrimp quality. In this respect, an increase of the shrimp pigmentation may turn into an increase in both the market value and acceptance of shrimp, especially regarding color upon cooking (Tume et al., 2009).

The present study was carried out to evaluate the potential to use MaB-flocs grown in an outdoor pilot reactor on pikeperch wastewater as ingredient for diets for shrimp. The technical feasibility was evaluated in growth-trials with juvenile Pacific white shrimp *Litopenaeus vannamei* in a hybrid recirculating aquaculture system. Five diets were formulated containing 0 to 8 % of dried MaB-flocs. To this aim, the shrimp growth (survival, growth, food conversion and size distribution) and quality (crude protein content, crude lipid content, fatty acid profile and pigmentation) were evaluated. Finally, the feasibility for industrial implementation of this concept is discussed with respect to diet costs and current European regulatory issues.

8.2. Materials and methods

8.2.1. MaB-flocs and preparation of diets

MaB-flocs were harvested during May-June 2013 from an outdoor pilot reactor treating wastewater from pikeperch culture (Aquaculture Practice Center at Inagro, Roeselare, Belgium). The reactor operation details are presented in Chapter 7. Upon settling by gravity, MaB-flocs were harvested and dewatered in linen filter bags (150-250 µm pore size; Lampe, Belgium) by a hydropress (4 bar; Enotecnica Pillan, Italy), dried at 105 °C and milled (all-grain stone mill, Landers Mora & CIA S., USA).

Based on the nutritional value of MaB-flocs (Table 8.1.; Table 8.2.; both presented in Section 8.3. Results and discussion), shrimp diets were formulated by CreveTec by replacing mainly wheat (0-49 %) by dried MaB-flocs (0, 2, 4, 6 and 8 % of the total diet), further referred to 0%MaB, 2%MaB, 4%MaB, 6%MaB, 8%MaB (Table 8.3.; presented in Section 8.3. Results and Discussion). In the reference diet, no immune stimulating products such as algae, seaweed or yeasts were added. Shrimp feed pellets were produced with a pellet mill on a 2 mm pore size screen including preconditioning with steam (>90 °C) and post-conditioning (>90 °C) during 20

minutes (Research Diet Services, The Netherlands). A combination of wheat flour and wheat gluten was used to obtain a good water stability of the shrimp feed pellets.

8.2.2. Shrimp, experimental units and feed management

The shrimp trial was performed at the CreveTEC - AFT Research Center (Venray, The Netherlands) over a 28-day period. There were 4 replicas of 31 shrimps for each of the 5 diets. The shrimp *L. vannamei* was selected for uniformity of size (6.56 ± 0.51 g) from an indoor shrimp farm (Movis, The Netherlands) and stocked in 20 nets of 150 L at a density of 31 per net (207 shrimps m^{-3}). All nets were placed in a bigger aerated tank in order to have the same water quality. Shrimp tanks temperature was maintained at 27-28 °C. The water quality in the shrimp tanks was maintained with a hybrid recirculation aquaculture system (RAS) utilizing a combination of heterotrophic bacterial flocs and clear water. This system consisted of aeration tanks, a buffer and settling tank, an oxidation/denitrification tank (without external addition of COD to the denitrificaton tank) with membrane filtration unit.

Throughout the 28-day growth trial, the water quality in the shrimp tanks was maintained at a suitable level for adequate growth and survival of the shrimps (Furtado *et al.*, 2011): 4.96 ± 0.67 mg DO L^{-1}, 14.86 ± 0.24 mg salinity L^{-1}, 79.1 ± 1.5 mg COD L^{-1}, 44.1 ± 21.5 mg soluble COD (SCOD) L^{-1}, 2.0 ± 13.2 mg TN L^{-1}, 0.16 ± 0.10 mg NH_4^+-N L^{-1}, 0.22 ± 0.23 mg NO_2^--N L^{-1}, 39.2 ± 5.2 mg NO_3^--N L^{-1}, 7.63 ± 1.24 mg TP L^{-1}, 7.63 ± 1.23 mg PO_4^{3-}-P L^{-1}, and had a temperature of 28.43 ± 0.57 °C, pH of 7.81 ± 0.29, EC of 16.82 ± 1.48 mS cm^{-1} (average of weekly measurements). None of these parameters showed a decreasing or increasing trend.

Each shrimp net was equipped with a continuous feeder (24 h, 5 kg, FIAP, Germany) which was filled daily. Feed gift (4.0, 3.8, 3.4 and 3.0 % of shrimp weight for week 1, 2, 3 and 4 respectively) was adjusted daily according to an expected growth curve (expected daily increase of 0.20 g day^{-1} for week 1, and 0.25 g day^{-1} for week 2, 3 and 4) based on the average shrimp weight from weekly initial and last measurements.

8.2.3. Analyses

MaB-flocs were analyzed for chlorophyll *a (*Chl*a)*, dry matter, ash free dry weight (AFDW) and ash content according to Van Den Hende et al. (2011a) and

carotenoid content according to Lichtenthaler (1987). The content of Ag, Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb and Zn of MaB-flocs was determined by ICP-OES (Vista-MPX, Varian, Australia) (APHA, 1998). The energy content (Vito, 2013), crude protein (EC, 1993), crude lipids (EC, 1998), fatty acid profile (AOAC, 1990), total amino acids (AOAC, 1993; Hugli and Moore, 1972; EC, 2009a) of MaB-flocs and diets were determined by Labo ECCA (Belgium).

Raw water samples of the shrimp tanks were weekly analysed for pH (VWR, Belgium), EC (K610, Consort, Belgium), DO (PCD650, Eutech Instruments, Singapore), total COD (APHA, 1998), TN and TP (Hach Lange, Belgium). Filtered (0.20 µm; RC-20125, Chromafil, Germany) water samples were analysed for NO_3^-, NO_2^-, NH_4^+, PO_4^{3-} (Hach Lange, Belgium) and soluble COD (APHA et al., 2005).

Each week, shrimps were counted and weighted to calculate the average shrimp weight in each net. After 28 days, each shrimp was also weighted separately. The survival (%) (100 x (final number of shrimps / initial number of shrimps), the total weight gain (g) (final weight – initial weight) and the feed conversion ratio (feeding gift / total weight gain) were calculated.

Dry weight, ash content, crude protein content, crude lipid content and fatty acid profile of raw muscle of shrimp fed with 0% and 8% MaB (3 samples of 15 shrimp from 3 nets for each per diet) were determined by Labo ECCA. Shrimp pigmentation was measured on cooked shrimp (2 min in 500 mL water of 100°C) by a chromameter (CAN 210, Japan). Reflectance values of lightness (L), redness (a) and yellowness (b) were measured on 10 cooked shrimp per diet on four locations of each shrimp abdominal segment (2^{nd} segment left side, 2^{nd} segment right side, 4^{th} segment left side; 4^{th} segment right side) and 3 measurements per location were made.

8.2.4. Statistical analyses

Statistical analyses was performed using PASW Stat.17.0 software (SPSS Inc., Chicago, USA). Data normality was determined with a Shapiro Wilk's test and homogeneity of variances with a Levene's test. If no normal distribution was observed, the differences in means were analysed by a Kruskal-Wallis with Mann-Whitney post-hoc test ($p < 0.05$) including a Bonferroni correction. Otherwise, one-way ANOVA with Tukey's post-hoc test was performed ($p < 0.05$).

8.3. Results and discussion

8.3.1. Harvesting and nutritional value of MaB-flocs

MaB-flocs were harvested from an outdoor MaB-floc pilot reactor which treated wastewater from pikeperch (*Sander lucioperca* L.) culture (Chapter 7). The settled MaB-flocs were dewatered by filtering at 150-250 µm in a pilot scale hydropress to a microalgal bacterial cake of 38.4 ± 6.1 % dry weight (DW) (weighted average; Table 8.1.). The dry weight of dewatered MaB-flocs increased in time which may be attributed to the increased ash content of the MaB-flocs (Table 8.1.).

The inorganic fraction of MaB-flocs mainly contained calcium (28.8 % of ash; Table 8.2.). If all calcium was precipitated as $CaCO_3$, this precipitate would be 71.8 % of the total ash content of MaB-flocs. MaB-flocs grown on calcium-rich wastewater can remove calcium as calcite crystals (Van Den Hende et al., 2012a). Calcium is an essential element for shrimp for shell hardening after molting, the hard tissue structure, blood clotting, muscle contraction, nerve transmission and osmoregulation as a cofactor for enzymatic procession of shrimp (Gunalan et al., 2013). Their calcium recommendation (RDA) is 0.5–2 % (Tacon, 1987). Many calcium supplements contain lead which impairs health in numerous ways (Gunalan et al., 2013). In contrast, the lead concentration in the calcium-rich MaB-flocs of this study was low (0.03 g Pb kg^{-1}). Next to lead, dried MaB-flocs contained (per kg) 1.07 g Fe, 0.46 g Zn, 0.21 g Al, 0.08 g Mn, 0.05 g Ba, 0.03 g Pb and 0.02 g B (Ag, Cd, Cr, Cu and Ni were under the detection limit of 0.01 g).

The relatively high ash content of MaB-flocs of this study resulted in a shrimp diet ingredient with a relatively low crude lipid, low crude protein content and low gross energy content compared to microalgae and bio-flocs (Table 8.2.). The crude lipid and protein content of MaB-flocs decreased with an increasing ash content, while the crude lipid and protein content of the AFDW of the MaB-flocs remained unaltered (Table 8.1.). This indicates that the crude lipid and protein content of the microorganisms did not vary in time. This is an interesting property for valorisation of extracts of proteins or lipids of MaB-flocs to for example aquaculture feed. In this study, the total biomass was used as shrimp diet ingredient including the ash. Compared to diatoms which also contain a large ash content due to the silica exoskelet and are often included in aquaculture feed for larvae (e.g., *Thalassiosira weissflogii* ;

Ju et al., 2009), the crude lipid content of MaB-flocs was higher, the protein content was similar, but the gross energy content lower (Table 8.2.).

The community structure of MaB-flocs was dominated by one microalgal species (*Ulothrix* sp. or *Klebsormidium sp.*; microscopic observation as presented in Chapter 7). The harvested MaB-flocs contained a low number of larvae of *Tubifex* sp. (around 10-20 per kg dewatered MaB-flocs). The Chl*a* content of the AFDW of MaB-flocs (Table 8.1.) was in the upper range of that of pure microalgae or cyanobacteria (0.17 to 4.36 %DW; Piorreck et al., 1984) and higher than earlier reports on the chlorophyll content of *Ulothrix* sp. (0.56 %DW; Jaya Prakash Goud *et al.*, 2007). This demonstrates the large content of microalgae in the MaB-flocs. Whereas several microalgal species are especially added to shrimp larvae diets because of their high content of highly unsaturated fatty acids such as eicosapentaoic acid (EPA) and docosahexaeonic acid (DHA), MaB-flocs did not contain a measurable amount of these fatty acids (Table 8.2.). In contrast, MaB-flocs contained much more α-linolenic acid (ALA) compared to other algae often used in aquaculture diets and compared to bio-flocs (Table 8.2.).

Table 8.1. *Composition of dewatered and dried MaB-flocs harvested on different days from an outdoor pilot reactor treating pikeperch wastewater in Roeselare, Belgium*

Compound		**MaB-flocs**		
		02/05/2013	**08/05/2013**	**12/06/2013**
After dewatering				
Dry matter	(%)	32.2	36.1	44.1
After drying				
Dry matter	(%)	99.2	99.6	99.2
Crude protein	(%DW)	27.7	24.9	15.8
Crude fat	(%DW)	4.40	3.90	2.64
Ash	(%DW)	50.3	55.8	70.2
Chlorophyll *a*	(%DW)	1.2	1.0	0.7
Ash free DW	(%DW)	49.7	44.2	29.8
Crude protein	(%AFDW[1])	56.6	56.8	53.9
Crude fat	(%AFDW)	9.00	8.90	8.99
Chlorophyll *a*	(%AFDW)	2.4	2.2	2.3
Gross energy[2]	(kJ g^{-1})	9.32	8.22	5.09
Digestable energy[3]	(kJ g^{-1})	8.06	7.13	4.46

[1] Ash free dry weight; [2] Gross energy content of the diets was calculated using kJ g^{-1} DW values of 21.3 for crude protein, 39.5 for crude fat and 17.2 for N-free extract; [3] Digestible energy for shrimps was calculated with the digestibility coefficients of 0.90 for crude protein, 0.95 for crude fat and 0.65 for N-free extract.

Table 8.2. *Composition of dried MaB-flocs from pikeperch wastewater treatment compared to bio-flocs, wheat, fishmeal, diatom T. weissflogii and microalgae Nannochloropsis sp.)*

Compound	**MaB-flocs[1]**	**Bio-flocs[2]**	**Wheat[3]**	**Fish meal[3]**	***T. weiss-flogii*[4]**	***Nanno-chlorop-sis*[4]**
Proximate and elemental composition (g kg^{-1})						
Dry matter	994.5	nd	973.0	915.0	848	909.2
Crude protein	209.9	280 – 420	107.5	715.0	216	319.3
Crude lipid	33.9	23 – 54	10.5	73.1	15.2	56.5
Ash	616.7	170 – 270	16.5	162.5	389	271
Ca	177.5	nd	0.7	35.0	Nd	nd
P	4.70	nd	3.3	25.0	Nd	nd
K	3.63	nd	4.5	13.0	Nd	nd
Mg	2.43	nd	1.1	2.3	Nd	nd
Na	0.48	nd	0.1	10.0	Nd	nd
Energy (kJ g^{-1})						
Gross energy calculated[5]	6.91	15.5 – 17.0	14.97	17.34	10.41	17.64
Gross energy measured[6]	7.06	nd	nd	nd	Nd	nd
Digestible energy[7]	6.01	nd	10.42	15.94	Nd	nd
Non-essential AA (g kg^{-1})						
Alanine	15.51	38.2	nd	nd	13.59	19.51
Aspartic acid + asparagine	25.13	63.6	nd	nd	26.78	21.46
Cysteine	3.18	5.5	2.34	5.15	1.14	1.62
Glutamic acid + glutamine	24.76	80.4	nd	nd	23.23	25.56
Glycine	14.49	28.1	nd	nd	11.68	17.82
Proline	8.56	27.7	nd	nd	16.95	29.45
Serine	8.93	28.2	nd	nd	9.00	12.19
Tyrosine	6.75	28.3	3.08	17.73	8.72	13.12
Total non-essential AA	107.31	300.0	nd	nd	111.09	140.73
Essential AA (g kg^{-1})						
Arginine	11.08	36.0	4.99	44.33	12.94	21.30
Histidine	2.61	14.6	2.04	17.52	3.63	5.20
Isoleucine	8.93	33.8	36.55	30.96	11.60	17.70
Leucine	15.63	50.6	7.42	56.63	12.70	23.29
Lysine	8.89	43.4	3.01	51.48	12.48	20.78
Methionine	4.67	14.1	1.76	22.17	5.02	6.42
Phenylalanine	10.32	32.9	4.73	30.67	10.97	18.05
Threonine	10.55	31.1	2.90	36.54	9.62	16.08
Tryptophan	3.06	9.8	nd	nd	3.28	2.32
Valine	12.63	35.2	nd	nd	11.82	20.56
Total essential AA	88.38	301.5	nd	nd	205.15	292.43
Fatty acids (g kg^{-1})						
C4:0	0.15	nd	nd	nd	Nd	nd
C6:0	<0.03	nd	nd	nd	Nd	nd
C8:0	0.07	nd	nd	nd	Nd	nd
C10:0	0.03	nd	nd	nd	Nd	nd
C12:0	0.37	nd	nd	nd	Nd	nd
C13:0	0.17	nd	nd	nd	Nd	nd

C14:0	1.36	0.1 - 0.6	nd	nd	2.01	2.67
C14:1	0.65	0.4 - 0.55	nd	nd	Nd	nd
C15:0	2.48	0.15 - 0.3	nd	nd	0.48	0.15
C15:1	<0.03	0.06 - 0.3	nd	nd	Nd	nd
C16:0	13.23	2.2 - 17	nd	nd	2.55	13.79
C17:0	0.71	0.05 - 0.4	nd	nd	3.17	0.23
C17:1	0.37	0.10 - 0.12	nd	nd	Nd	nd
C18:0	1.56	0.5 - 6.0	nd	nd	0.08	0.47
C18:1c	4.73	nd	nd	nd	Nd	nd
C18:1(n-9)	nd	1.8 - 18	nd	nd	0.10	2.71
C18:1(n-7)	nd	1.5 - 3.0	nd	nd	0.07	0.45
C18:2(n-6) (LA)[8]	2.41	5 – 19	0.00	9.14	1.82	2.54
C18:3(n-3) (ALA)[9]	4.05	0.04 - 2	0.00	3.66	0.07	0.15
C18:3(n-6) (GLA)[10]	<0.03	0.15 - 0.4	nd	nd	0.02	0.51
C18:4(n-3) (STA)[11]	<0.03	0.16 - 0.25	nd	nd	0.08	0.03
C20:0	0.48	0.1 - 0.2	nd	nd	0.00	0.00
C20:1	<0.03	0.05 - 0.10	nd	nd	Nd	nd
C20:2(n-6)	<0.03	nd	nd	nd	0.00	0.00
C20:3(n-3)	<0.03	0.00	nd	nd	0.03	0.02
C20:3(n-6)	<0.03	0.15 - 0.07	nd	nd	Nd	nd
C20:4(n-3)	<0.03	0.10	nd	nd	0.00	0.00
C20:4(n-6) (AA)[12]	<0.03	0.2 - 0.7	nd	nd	0.02	1.81
C20:5(n-3) (EPA)[13]	<0.03	0.10 - 0.25	0.00	5.85	2.22	0.00
C22:0	0.31	0.14 - 0.15	nd	nd	0.01	nd
C22:1	<0.03	0.1	nd	nd	Nd	nd
C22:5(n-3) (DPA)[14]	<0.03	0.05	nd	nd	0.00	0.00
C22:6(n-3) (DHA)[15]	<0.03	0.05	0.00	3.66	0.32	0.00
C24:0	<0.03	0.00	nd	nd	Nd	nd
C24:1	<0.03	0.09 - 0.3	nd	nd	Nd	nd
∑ SFA[16]	20.94	nd	nd	nd	6.29	14.72
∑ MUFA[17]	6.56	nd	nd	nd	Nd	nd
∑ PUFA[18]	6.49	nd	nd	nd	3.06	20.05
∑ HUFA[19]	<0.18	nd	nd	nd	2.64	1.84
Total n-3	4.05	0.4 - 0.65	nd	nd	Nd	nd
Total n-6	2.41	7 - 20	nd	nd	Nd	nd
n-6/n-3	0.595	18	nd	nd	Nd	nd

[1] Mix of dried MaB-flocs of several harvests: 1201 g of 02/05/2013, 771 g of 08/05/2013, 2145 g of 12/06/2013; [2] Ranges for freshwater bioflocs (Crab et al., 2010), except for AA profile (Logan et al., 2010); [3] Wheat and fish meal used in this study; [4] Ju et al., 2009; [5] Calculated from the biochemical composition; [6] Calorific bomb; [7] Calculated for shrimp with digestibility coefficients of 0.95 for crude lipid, 0.9 for crude protein and 0.65 for N-free extract; [8] Linolenic acid; [9] Alpha linolenic acid; [10] Gamma linolenic acid; [11] Stearodonic acid; [12] Arachidonic acid; [13] Eicosapentaenoic acid; [14] Docosapentaenoic acid; [15] Docosahexaenoic acid; [16] Saturated fatty acids; [17] Mono unsaturated fatty acids; [18] Poly unsaturated fatty acids; [19] Highly unsaturated fatty acids: minimum 4 double bounds; nd: no data.

8.3.2. Diet formulations

Several shrimp diets were formulated by replacing other ingredients by MaB-flocs while aiming at iso-nitrogenous and iso-lipidic diets. In most of the studies in which algae or bio-flocs are employed in shrimp diets, they usually replace fishmeal because of its high cost in shrimp diets and limited resource (Anand et al., 2013; Bauer et al., 2012; Ju et al., 2009). In our study, replacing fish meal by MaB-flocs was not recommendable for several reasons. Firstly, it would not be possible to formulate iso-nitrogeneous shrimp diets, because of the low crude protein content of MaB-flocs which was 3.4 times lower compared to fishmeal (Table 8.3.). Secondly, it would be hard to formulate iso-lipidic diets and iso-energetic diets by replacing fishmeal by MaB-flocs. MaB-flocs showed a 2.2 times lower crude lipid content, 2.5 times lower gross energy and 2.7 times lower digestible energy compared to fishmeal (Table 8.2.). Thirdly, the P:Ca ratio in shrimp diets needs to be balanced (D'Abramo et al., 1997; Table 3). MaB-flocs showed a very low P:Ca of 0.026 and contained 5.1 times more calcium compared to fishmeal (Table 8.2.).

MaB-flocs were used to substitute mainly wheat (13.97 to 48.61 %), a shrimp diet ingredient with a relatively low crude protein and digestible energy content, for 2 to 8 % of the total diet (Table 8.3.). Compared to wheat, MaB-flocs contained 2 times more crude protein and 3 times more crude lipid but a 1.7 times lower digestible energy content (Table 8.2.). Diets composition were based on *L. vannamei* requirement for proteins, methionine, cysteine, lysine, arginine, lipids, highly unsaturated fatty acids (HUFA), phospholipids and cholesterol of (adapted from d'Abramo et al., 1997). To reach these requirements for all diets, fish oil, soy lecithin, fish meal and soy bean meal were varied little among the diets (Table 8.3.). The protein content of the screened diets ranged from 37.99%DW to 39.29 %DW and the lipid content from 6.98 %DW to 6.85 %DW (Table 8.3.; presented in Section 8.3. Results and Discussion). The addition of MaB-flocs to the shrimp feed, led to light to deep green feed pellets, compared to brown pellets of the reference diet.

Table 8.3. *Formulations and composition of diets with varying levels of MaB-flocs*

	MaB-floc inclusion				
	0 %	**2 %**	**4 %**	**6 %**	**8 %**
Ingredients (%)					
Danish fish meal LT[1]	25.00	26.00	26.00	26.00	26.00
Wheat flour[2]	25.00	25.00	25.00	25.00	25.00
Wheat[3]	17.32	14.90	12.90	10.90	8.90
Soybean meal[4]	15.40	15.00	15.00	15.00	15.00
Corn gluten[4]	5.00	5.00	5.00	5.00	5.00
MaB-flocs[5]	0.00	2.00	4.00	6.00	8.00
Rice bran[6]	5.00	5.00	5.00	5.00	5.00
Wheat gluten[4]	4.00	4.00	4.00	4.00	4.00
Fish oil[7]	2.40	2.00	2.00	2.00	2.00
Squid meal[8]	2.00	2.00	2.00	2.00	2.00
Soy lecithin[9]	1.78	1.95	1.90	1.85	1.80
Vitamin premix[10]	1.00	1.00	1.00	1.00	1.00
Mineral premix[10]	1.00	1.00	1.00	1.00	1.00
Cholesterol[11]	0.10	0.10	0.10	0.10	0.10
Share of compound replaced by MaB-flocs (%)					
Wheat	0.00	13.97	25.52	37.07	48.61
Fish oil	0.00	16.67	16.67	16.67	16.67
Composition (% DW)					
Protein	37.99	38.67	38.88	39.08	39.29
Lipids	6.98	6.85	6.85	6.85	6.85
Methionine	0.90	0.93	0.93	0.94	0.95
Methionine + cysteine	1.39	1.41	1.42	1.43	1.44
Lysine	2.14	2.19	2.20	2.21	2.22
Arginine	2.23	2.25	2.25	2.24	2.23
HUFA[12]	0.76	0.69	0.69	0.69	0.69
Phospholipids	1.50	1.65	1.65	1.65	1.66
Cholesterol[13]	0.20	0.20	0.20	0.20	0.20
Ash	6.85	8.19	9.39	10.58	11.78
Ca	0.95	1.33	1.69	2.04	2.39
Mg	0.34	0.35	0.35	0.35	0.35
Na	0.30	0.31	0.31	0.31	0.31
K	0.91	0.91	0.91	0.90	0.90
P	1.07	1.10	1.10	1.10	1.11
P:Ca	1.13	0.83	0.65	0.54	0.46
Gross energy (kJ g^{-1})[14]	20.90	20.48	20.18	19.87	19.57
Digestable energy (kJ g^{-1})[14]	16.73	16.25	15.96	15.67	15.38

Notes with Table 8.3.

Shrimp (*L. vannamei*) diets were commercially manufactured by Research Diet Services (The Netherlands) using extrusion processing. [1] Triple nine, Denmark; [2] Meneba, The Netherlands; [3] Van Eck, EU; [4] Cargill, The Netherlands; [5] MaB-flocs were harvested during spring 2013 from a pilot reactor treating pikeperch wastewater in Belgium; [6] Vitalia, country of origin not known; [7] INVE, Belgium and Smit Weesp, The Netherlands, Brand/supplier, country; [8] Seah International, France; [9] Cargill, country of origin not known; [10] CreveTec, Belgium; vitamin and mineral premixes are proprietary products thus their composition is not listed; [11] Disman, The Netherlands; [12] Highly unsaturated fatty acids: minimum 4 double bounds; [13] Assuming no significant amount of cholesterol is present in MaB-flocs; [14] Gross energy was calculated using kJ g^{-1} DW values of 21.3, 39.5 and 17.2 and digestible energy via digestibility factors of 0.90, 0.95 and 0.65, for crude protein, crude lipid and N-free extract respectively.

8.3.3. Shrimp survival and growth

The survival and growth of *L. vannamei* were not significantly affected by including 2 to 8 % MaB-flocs in its diets (Table 8.4.). The shrimp survival was high among all diets (Table 8.4.; Bauer et al., 2012). No significant differences among diets were observed in the weekly measured shrimp weight (Fig. 8.1.a). The average daily weight gain did not significantly differ among diets (Table 8.4.). This weight gain was up to 3 times higher compared to other studies with a similar final shrimp weight (Ouijifard et al., 2012; Xu et al., 2012; Cruz-Suárez et al., 2010). This fast shrimp growth supports the beneficial shrimp growth conditions of the used hybrid RAS system and feeding regime with continuous feeding. The addition of MaB-flocs did not affect the distribution of the final shrimp weights (Fig. 8.2.). While other studies demonstrated an increase in the shrimp growth by the inclusion of microalgae in diets of *L. vannamei* in longer trials (Ju *et al*., 2009), this was not observed in this study.

Whether the shrimp growth increases by adding algae to the shrimp diets, depends on the algal species (Ju et al., 2009). To the best of our knowledge, no studies have been published on the effect of MaB-flocs, *Ulothrix* sp. or *Klebsormidium sp.* on shrimp growth. In our study, a possible negative effect of an increased ash content of the alternative feed diets (Table 8.3.), could have masked the possible positive effect of algae to the shrimp growth in our study. Therefore, future growth trials are needed to distinguish the effect of ash and microalgae present in MaB-flocs on the shrimp growth and to study their effect on stressed shrimps.

Table 8.4. *Growth performance of juvenile shrimp L. vannamei fed diets with varying levels of MaB-flocs*

MaB-flocs in diet (%)	**Survival[1] (%)**	**Initial weight[1] (g)**	**Final weight[1] (g)**	**Total weight gain[1] (g)**	**Average weight gain[1] (g day^{-1})**	**FCR[2]**	**Final net yield[1] (g m^{-3})**
0	94.4 ± 1.6	6.47 ± 0.19	15.98 ± 0.57	9.52 ± 0.69	0.34 ± 0.02	1.21 ± 0.07	3118 ± 166
2	96.0 ± 3.1	6.13 ± 0.18	15.60 ± 0.50	9.47 ± 0.43	0.34 ± 0.02	1.17 ± 0.02	3095 ± 182
4	92.7 ± 4.8	6.63 ± 0.35	15.86 ± 0.47	9.23 ± 0.28	0.33 ± 0.01	1.27 ± 0.06	3040 ± 192
6	96.0 ± 1.6	6.70 ± 0.50	15.64 ± 1.26	8.94 ± 0.78	0.32 ± 0.02	1.23 ± 0.08	3100 ± 230
8	96.8 ± 2.6	6.90 ± 0.86	16.25 ± 0.56	9.35 ± 0.57	0.33 ± 0.02	1.22 ± 0.10	3250 ± 171

No significant differences of values within the same column were found ($p < 0.05$). [1] Mean ± standard deviations of 4 replicates containing 31 shrimp/tank; [2] Feed conversion ratio

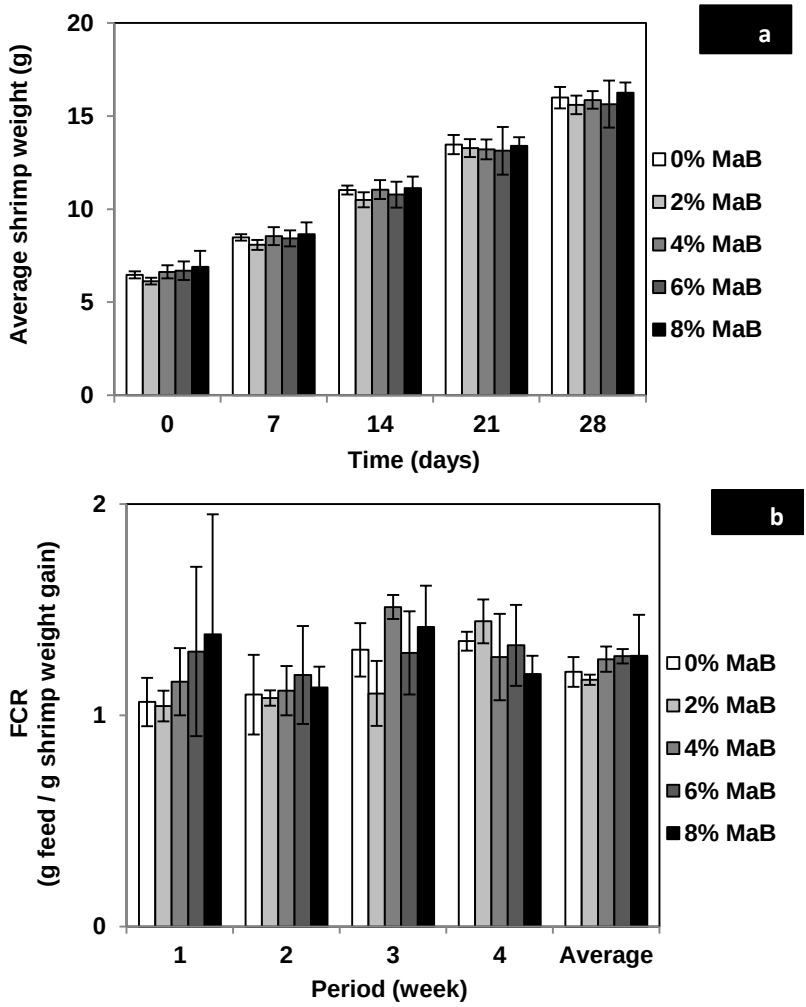

Fig. 8.1. *Weekly variations in average shrimp weight (a) and food conversion rate (b) of shrimp L. vannamei for diets with varying levels of MaB-flocs*
No significant differences of values within the same time or period were found ($p < 0.05$)

The final FCR did not significantly differ among diets (Table 8.4.) and were similar to better as compared to other studies with a similar final shrimp weight resulting in a FCR of 1.51-1.85 (Bauer et al., 2012), 1.14-2.12 (Oujifard et al., 2012), 1.25-1.33 (Sookying et al., 2011). No clear trend was observed in the weekly FCRs (Fig. 8.1.b). The increased ash content of the MaB-floc containing diets did not affect the FCRs. A decreased P:Ca content of the diets can be disadvantageous for the shrimp growth, but on the other hand the addition of carbonate salts to the shrimp tanks can be beneficial in bio-floc technology shrimp cultures in order to maintain the water

alkalinity of the shrimp cultures between 100 and 150 mg L^{-1} (Furtado *et al*., 2011). During our 28 day-trial, maximum 50 mg $CaCO_3$ L^{-1} originating from MaB-flocs was added to the shrimp tanks of which a part might have been converted to CO_2.

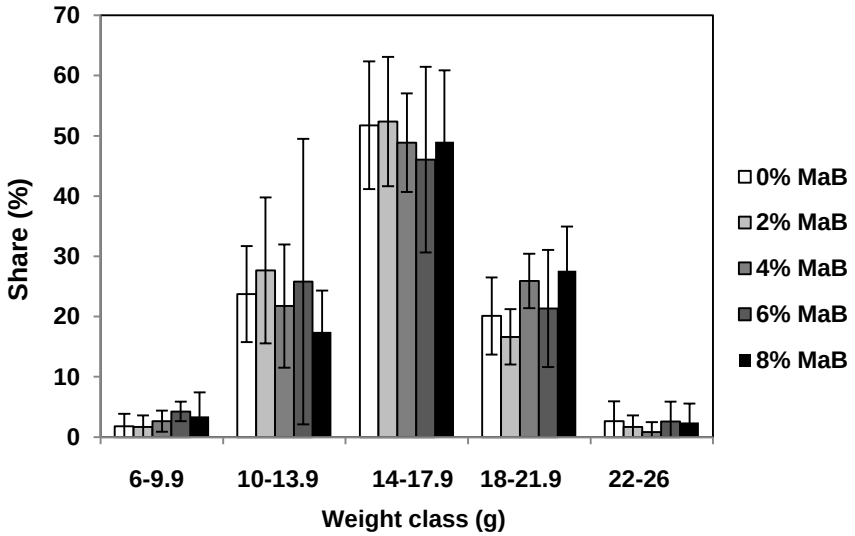

Fig. 8.2. *Final size distribution of shrimp L. vannamei fed with diets with varying levels of MaB-flocs*
No significant differences of values within the same weight class were found ($p < 0.05$).

8.3.4. Shrimp color and nutritional value

Increasing the amount of MaB-flocs in shrimp diets from 0 to 8 % significantly increased the reflectance of redness from 15.06 ± 1.04 to 19.37 ± 1.85 and of yellowness from 20.98 ± 1.63 to 24.47 ± 1.36 of cooked shrimp tails (Fig. 8.3). This reflectance of redness was positively correlated with the MaB-floc content in their diets ($R^2 = 0.9865$; following a linear model within the ranges tested $y = 0.5234x + 15.296$). The addition of only 4 % of MaB-flocs already significantly increased this redness (Fig. 8.3.). The pigmentation in shrimp is attributed to the presence of carotenoids, mainly astaxanthin (Parisenti et al., 2011). Carotenoids are also a source of provitamin-A, increase survival rate and weight gain, enhance resistance against the white spot syndrome virus, maintain resistance to stress and ammonia excess, improve hepatopancreatic function, provide anti-oxidant protection, protect cholesterol and polyunsaturated fatty acids from oxidation (Parisenti et al., 2011 and references herein).

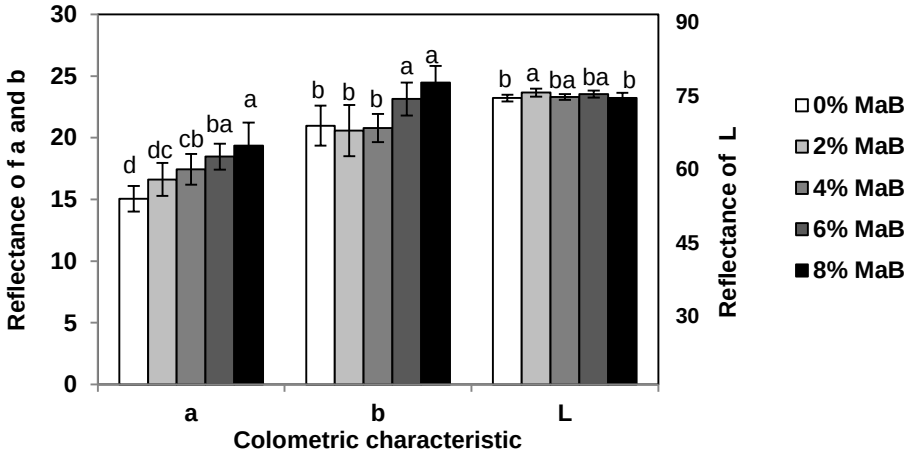

Fig. 8.3. *Reflectance of cooked shrimps L. vannamei fed with diets with varying levels of MaB-flocs: a (redness), b (yellowness) and L (luminosity)*
Mean ± standard deviations of 120 measurements per diet (10 cooked shrimps per diet with 4 locations per shrimp and 3 measurements at each location: 2nd tail segment left, 2nd tail segment left, 4th tail segment right, 4th tail segment left). Significant differences according to one-way ANOVA and Tukey's post-hoc test are indicated with a different letter ($p < 0.05$).

MaB-flocs of this study contained 2.52 mg carotenoids g^{-1} DW or 6.63 mg g^{-1} ash free dry weight. This is similar to *Ulothrix* species (4.5 mg carotenoids g^{-1}) and high compared to several other freshwater microalgae (Jaya Prakash Goud et al., 2007). The presented results suggest that these carotenoids from MaB-flocs can be efficiently assimilated, metabolized and converted to astaxanthin by shrimp. Including 8 % MaB-flocs to shrimp diet, means including 0.14 mg carotenoids originating from MaB-flocs per g of diet or 531 µg of carotenoids g^{-1} shrimp biomass over the 28-day trial. A content of 60 µg carotenoids g^{-1} shrimp biomass can already lead to an increased pigmentation of *L. vannamei* (Ju et al., 2009). This enhanced pigmentation can increase the market value of shrimp, especially upon cooking because the red/orange shrimp color is associated with freshness and product quality (Parisenti, 2011; Cruz-Suarez et al., 2010).

The content of crude lipid and protein of raw shrimp muscle were not significantly affected by the inclusion of 8 % MaB-flocs in the diet (Table 8.5.) and were similar to previous studies (Gunalan et al., 2013 and references herein; Zhou et al., 2012).

Table. 8.5. *Proximate composition and fatty acid profile of shrimp muscle of L. vannamei fed on diets with 0 and 8 % of MaB-flocs*

Compound	**MaB-flocs**	
	0 %	**8 %**
Proximate composition (g kg^{-1})		
Dry matter	244 ± 4	247 ± 4
Crude protein	215 ± 5	219 ± 3
Crude lipid	12 ± 1	12 ± 1
Ash	15.3 ± 0.4	15.7 ± 0.1
Fatty acids (mg kg^{-1})		
C10:0	16 ± 28	<1
C12:0	36 ± 36	15 ± 13
C14:0	149 ± 69	89 ± 15
C15:0	16 ± 28	31 ± 27
C16:0	2748 ±114	2651 ± 97
C16:1	194 ± 3	178 ± 10
C17:0	64 ± 55	97 ± 10
C18:0	1038 ± 51	1015 ± 41
C18:1t	51 ± 20	43 ± 6
C18:1c	2517 ± 147	2545 ± 253
C18:2t	31 ± 8	27 ± 5
C18:2(n-6) (LA)[1]	1464 ± 122	1506 ± 97
C18:3(n-3) (ALA)[2]	176 ± 56	187 ± 38
C20:0	35 ± 10	35 ± 13
C20:1	80 ± 69	125 ± 13
C20:2(n-6)	152 ± 8	156 ± 13
C20:3(n-3)	16 ± 14	23 ± 1
C20:4(n-6) (AA)[3]	175 ± 6	183 ± 13
C20:5(n-3) (EPA)[4]	1286 ± 33	1377± 95
C22:0	42 ± 22	31 ± 14
C22:5(n-3) (DPA)[5]	62 ± 3	58 ± 12
C22:6(n-3) (DHA)[6]	1233 ± 60	1302 ± 109
∑ SFA[7]	4191 ± 239	3961 ± 144
∑ MUFA[8]	2827 ± 142	2844 ± 245
∑ PUFA[9]	4566 ± 262	4793 ± 307
∑ HUFA[10]	2755 ± 91	2921 ± 219
Total n-3	2772 ± 138	2948 ± 213
Total n-6	1790 ± 119	1841 ± 128
n-6/n-3	0.645 ± 0.014	0.625 ± 0.036

[1] Linolenic acid; [2] Alpha linolenic acid; [3] Arachidonic acid; [4] Eicosapentaenoic acid; [5] Docosapentaenoic acid; [16] Docosahexaenoic acid; [7] Saturated fatty acids; [8] Mono unsaturated fatty acids; [9] Polyunsaturated fatty acids; [10] Highly unsaturated fatty acids: 4, 5 or 6 double bounds.

The following fatty acids were < 0.1% for both diets: C4:0, C6:0, C8:0, C11:0, C12:1, C13:0, C14:1, C17:1, C18:3(n-6), C18:4 (n-3), C20:3 (n-6), C20:4 (n-3), C22:1, C24:0, C24:1.

No significant differences of values within the same row were observed ($p < 0.05$).

Whereas the 8% MaB-floc diets contained nearly twice as much ash as compared to the reference diet (Table 8.3.), the shrimp muscle from 8%MaB-floc diets

did not show a higher ash content compared to the reference diet (Table 8.5.). Diets containing 8 % MaB-flocs did not significantly modify the fatty acid profile of the shrimp muscle compared to the reference diet. The levels of EPA and DHA of shrimp muscle were not significantly affected, despite the 10% lower EPA (0.35 %DW *vs* 0.39 %DW resp.) and 9 % lower DHA (0.30 %DW *vs* 0.33 %DW resp.) content of 8%MaB diets compared to the reference diet. EPA and DHA may have been produced by the elongation of the essential fatty acid ALA, highly present in MaB-flocs (Table 8.2.). In addition, an increased level of carotenoid originating from MaB-flocs in the 8%MaB-floc diet may have served as anti-oxidant to protect the available HUFA and in this way influenced the HUFA content in shrimp positively. Similarly, Cruz-Suarez et al. (2010) observed an increased EPA and DHA content in the lipid fraction of muscle of *L. vannamei* fed with macroalga *Ulva* containing low amounts of EPA and DHA and attributed this to the elongation of linolenic acid (LA) highly present in *Ulva* sp.

8.3.5. Industrial application: feed costs and regulation issues

Previous results demonstrate that the inclusion of MaB-flocs fed on aquaculture wastewater in shrimp diets is technical feasible. However, whether their inclusion will be applied on industrial scale, will greatly depend on the feed costs and on the limits set by legislation regulations, next to the future availability and composition of MaB-flocs.

The costs for the raw materials of the alternative diet containing 8% MaB-flocs will not be higher compared to the reference diet if MaB-flocs can be dried and grinded for 100 € per ton prepared biomass (Table 8.6.). Nevertheless, this value of 100 € per ton is most probably not feasible (in west-European countries). Drying consumes 70-110 kWh m^{-3} water for conventional thermal drying systems and 22-28 kWh m^{-3} water for solar based dewatering systems (Bux et al., 2002). Drying the dewatered MaB-flocs (worst case scenario of 32.2 % DW; Table 8.1.) means removing of 2.1 m^3 of water per ton dried MaB-floc biomass or 22-36 € per kg conventional dried MaB-flocs and 7-9 € per ton solar dried MaB-flocs (if 0.15 € kWh^{-1}). Per ton dried biomass, dewatering with a belt filter press costs 400-700 kWh or 60-105 € (Udom et al., 2013). However, no costs for harvesting and dewatering are taken into account because these are inherent to the wastewater treatment and present no

extra cost. Considering all other costs such as grinding and man labor, a more realistic price drying and grinding MaB-flocs (in Europe) will be around 200-300 € per ton which is in the same order as wheat (200-220 € per ton; 09/2013). In this case, the cost for raw materials of the alternative diet will be 8-16 € per ton higher than the reference diet (Table 8.6.). This extra cost is very low compared to the current high prices for shrimp (4,000-10,000 € per ton; July, 2013) and may be balanced by the added market value due to the enhanced red pigmentation. In integrated aquaculture systems, fresh MaB-flocs originating from treatment of fish culture effluent could fed to shrimp cultures. In this way, there would be no costs of drying MaB-flocs.

Future investigation is needed to proof if the costs of MaB-floc-containing diets for shrimp can be lowered by replacing more high cost ingredients such as fish oil by MaB-flocs. Furthermore it should be investigated to which extent the MaB-floc inclusion in shrimp diets can be increased while further increasing the shrimp pigmentation but without decreasing the shrimp productivity and the shrimp carcass quality.

Current regulatory issues limit the use of wastewater-grown MaB-flocs in shrimp diets in certain countries. This is particularly the case in all EU Member States since the addition of wastewater grown algae in animal feed is subject to tight EU regulations. Recently, algae were added to the European Catalogue of feed materials (regulation (EC) No 68/2013; EC, 2013b) and can now be included in animal feed in the EU market if they contain less than 0.1% of antifoaming agents. However, the inclusion of MaB-flocs (and also algae in general) grown on aquaculture wastewater in animal feed is restricted in Europe by regulation (EC) No 767/2009 (EC, 2009b). Firstly, this regulation restricts the use of waste from treatment of industrial wastewater in animal feed. However, EU Directive 91/271/EEC (EC, 1991) defines industrial wastewater as 'wastewater which is discharged'. This means that if aquaculture process water treated by MaB-flocs is reused again in the same company and thus not discharges, it should not be considered as wastewater. So, if MaB-flocs are part of a recirculating aquaculture system (RAS), they could be included in feed in the European market. Secondly, regulation (EC) No 767/2009 restricts the use of faeces and urine including of fish (aquaculture). Therefore, MaB-flocs should be proven to be free of faeces and urine before they can be included in feed for fish or shrimp. Interestingly, cultured fish and shrimp for the European food market do not

have to be free of urine or faeces. Moreover, in other European regulations exceptions are made for faeces and urine of fish. For example, regulation (EC) No 1069/2009 (EC, 2009c), restricting the use for animal feed of animal by-products directly retained from manure or treatment sludge, does not apply to faeces or urine of fish (aquaculture). So, if fish urine and faeces would be excluded from annex 3 of regulation (EC) No 767/2009, the use of MaB-flocs from process water of a RAS in feed in the European market would not be restricted by current European legislation. Next to economic and legislation analyses, more scientific research is needed before MaB-flocs should be included in shrimp feed diets. Especially the impact of the proposed MaB-floc-feed-shrimp-food concept on the environment (e.g. greenhouse gas emissions; effluent quality regarding toxins, suspended algae when process failure and pathogen removal; energy-efficiency), health of MaB-floc-consuming animals (e.g. compounds affecting animal health such as toxins, heavy metals and pathogens) and human health (food safety regarding toxins, heavy metals, pathogens) needs to be assessed scientifically. This may include a life cycle analysis (LCA).

Table 8.6. *Costs of raw materials of the MaB-floc containing diet formulations compared to the reference diet*

	Unit	**Refe-rence diet**	**MaB-flocs (%)**			
			2	**4**	**6**	**8**
Max. cost MaB-flocs[1]	(€/ton)	/	-288,5	-27.80	59.20	102.70
Costs of raw materials for feed if MaB-flocs cost						
1.Case 1: 100 €/ton	(€/ton)	977.78	985.25	982.59	979.93	977.27
2.Case 2: 200 €/ton	(€/ton)	977.48	987.25	986.59	985.93	985.27
3.Case 3: 300 €/ton	(€/ton)	977.48	989.25	990.59	991.93	993.27

[1] Maximum cost of MaB-flocs in diet formulations containing 2 %, 4 %, 6 % or 8 % MaB-flocs to obtain the same cost as for the reference diet.

In contrast, in case if MaB-flocs are grown on process water from food industry and if the effluent from the MaB-floc reactor is reused again, the regulations are less severe. According to regulation (EC) No 767/2009, food company process water which contains materials from food or feed and which is free from disinfectants, cleaning products or other products which are not allowed by the European animal feed legislation, can be directly used for animal feed. In this case, MaB-flocs grown on this reused process water can also enter the European feed market.

It should be noted that the import of shrimps grown on market feed containing aquaculture wastewater fed MaB-flocs is not directly restricted by legislation (if shrimp quality meets all the other European food standards). This means, that if the future society will demand for a more sustainable aquaculture industry including recovery of nutrients and by-products, the current European regulations can be disadvantageous for European aquaculture companies not applying RAS systems and advantageous for countries where the latter is approved.

8.4. Conclusions

MaB-flocs originating from treatment of pikeperch culture wastewater, can act as ingredient of diets of white pacific shrimp *L. vannamei*. Indeed, notwithstanding the high ash content of MaB-flocs, the inclusion of MaB-flocs in shrimp diets for up to 8 % did not affect the survival, growth and food conversion rate of shrimp, nor the proximate composition and fatty acid profile of shrimp muscle. However, the inclusion of MaB-flocs in shrimp diets significantly increased the redness and yellowness of tails of cooked shrimp. Nevertheless, the implementation of this promising integrated aquaculture concept in Europe calls for an adjustment of current European regulations. Future research should screen increased levels of MaB-flocs in shrimp diets.

8.5. Acknowledgements

This study was realized with financial support from a Ph.D. award from the Ernest du Bois Fund managed by the King Baudouin Foundation. MaB-floc production was supported by the INTERREG IVB NWE program, the Flemish Government, Province West-Flanders and University College Flanders (Howest) within the project EnAlgae. The authors thank Inagro for providing wastewater; B. Frese (AFT) and G.Bore (EME) for their help with shrimp weighting; N. Beernaert (ILVO) for the use of the chromameter; Q. Dumont de Chassart and J. Van Nevel (Federal Government Department Health) for critically revising the part on European regulations; J. Van Wichelen (UGent) for microscopic determinations; L. Van Den Hende (VEBA) for shrimp sample preparations; R. De Weirdt and A. Scoma (UGent) for critically reading the manuscript.

Yes,

but no.

(Bore, 2013)

Picture on previous page:

Outdoor MaB-floc raceway pond treating pikeperch culture wastewater located at Inagro in Roeselare.

Quote on previous page:

Gaëlle Bore, a master student from EME, summarizing the feasibility of MaB-floc ponds in 3 words at the end of her EnAlgae internship.

CHAPTER 9

Discussion and perspectives

9.1. Introduction

Sunlight-powered microalgal bacterial systems for wastewater treatment (MaB-S-WWT) based on photosynthetic aeration have large potential. However, as reviewed in **Chapter 1**, several challenges hamper the implementation of MaB-S-WWTs. A major challenge is the separation of the treated wastewater from the microorganisms. To address this, we presented a novel concept: microalgal bacterial flocs in sequencing batch reactors (MaB-floc SBRs). This concept was screened in lab-scale reactors for treatment of various wastewaters and was up-scaled to an outdoor raceway pond for aquaculture wastewater treatment.

The currently widely applied conventional activated sludge system (CAS) for wastewater treatment has been described in 1913 (Ardern and Lockett, 1914) and is still being optimised one century later (Jassby et al., 2014). Compared to CAS technology, the MaB-floc SBR technology presented in this dissertation is very new. It is thus not a cliché to state that further optimisation of MaB-flocs SBRs is needed before it will be implemented on industrial scale. To identify the critical research needs for MaB-floc SBRs, this chapter presents an overview of the features, limitations and research needs of MaB-floc SBRs (Table 9.1-3.). This overview is based on the research outcomes and insights provided in this work, but also on hands-on-experience and valuable discussions with various stakeholders. Furthermore, future perspectives for MaB-floc technology are outlined.

9.2. Key features, limitations and research needs of MaB-floc SBRs

9.2.1. Bioflocculation: easy and effective, but mechanisms to be revealed

MaB-flocs were developed via bioflocculation of microalgae. A consortium of microorganisms - including microalgae - were collected from natural ponds and at wastewater treatment plants (**Chapter 2, 3, 5, 6 and 7**). Especially periphyton that naturally grows on reactor walls, grids, settling tanks and in ponds provide ‘a free market’ of MaB-floc inoculum. This means that there is no need to spend energy and time in buying or developing and maintaining axenic monocultures. In 1-2 weeks, adequate settling MaB-flocs were obtained in sewage and 5 different industrial wastewater types by sequential stirring and settling of the inoculum followed by SBR operation mode. The wide availability and cheap source of inoculum and the simple

and fast MaB-floc formation protocol lower the barrier for further research by all interested stakeholders and for (agro-)industrial implementation.

Bioflocculation enabled discharge of biomass-free effluent (**Chapter 2, 3, 5, 6 and 7**). This was obtained without the use of additional biomass removal technologies and without contaminating the effluent with flocculants. This is a major advantage. A decreased OC:IC ratio of the wastewater decreased the MaB-floc settling due to the dominance of filamentous cyanobacteria (**Chapter 2**). This finding was confirmed in batch experiments with various (agro-)industrial wastewaters (**Chapter 6**). The addition of organic carbon in wastewaters with an unbalanced OC:IC ratio might be too expensive. Another strategy, as demonstrated in paper mill wastewater treating MaB-flocs (**Chapter 5**) and in the outdoor raceway MaB-flocs (**Chapter 7**), is calcium-containing crystal formation within the MaB-floc. This allows fast settling of MaB-flocs despite the dominance of filamentous microorganisms. To avoid bulking sludge due to oxygen accumulation, the effluent should be withdrawn after a long dark period, e.g., at the end of the night (**Chapter 6**). To avoid colonization of the reactor liquor by suspended microalgae, covering of the influent tank is of large importance, as observed in the MaB-floc raceway pond (**Chapter 7**).

Despite the fast and successful MaB-floc formation in various wastewaters (**Chapter 2, 3, 5, 6 and 7**), the mechanisms underlying bioflocculation remain to be revealed. MaB-flocs are still a black box microcosm with respect to MaB-floc architecture (e.g., anaerobic, anoxic and aerobic zones; nutrient diffusion; EPS), community structure (richness, organization and dynamics) and functionalities. To quantify the total cell volume or total cell count of microalgae and bacteria in the MaB-floc, molecular fingerprinting and microscopy have been applied (Park et al., 2011a; Szabo et al., 2008; Oron et al., 1979). Further optimisation is needed to develop easier methods for this. Furthermore, the robustness and resilience of MaB-flocs and the effect of bioflocculation on wastewater treatment and biomass production need further study. The MaB-floc field lies open to specialists in ‘omics’ and advanced microscopy to determine ‘who is doing what with whom when and where’ in MaB-flocs as a first step towards further process optimisation by microalgal and bacterial resource management.

9.2.2. Reactor operation: as simple as possible but not any simpler

During this study, the proof-of-concept of MaB-floc SBRs was demonstrated on lab scale for treatment of various wastewaters and was confirmed for one wastewater type in an outdoor raceway pond (**Chapter 7**). No major problems were observed during start-up of this pond in harsh winter conditions and during reactor operation from winter till autumn in Northwest Europe.

MaB-floc reactors are operated as SBR, which is a well-known strategy to develop fast settling microbial flocs via selection of 'the fastest settling' (Tchobanoglous et al., 2003). One of the major advantages of SBRs compared to continuous stirred reactors with settling tank, is the absence of a settling tank in an SBR set-up (Cervantes et al., 2006). Nevertheless for MaB-floc harvesting, a settling tank is needed. The estimated area of this settling tank would be around 1 % of the pond reactor area in case of the studied aquaculture wastewater. Whereas SBRs are often characterized by a less stable reactor performance (Tchobanoglous et al., 2003), this problem was not observed in this study (**Chapter 2, 3, 5, 6 and 7**). Compared to continuous stirred reactors with settling tanks, SBR operation requires a higher level of sophistication for timing units and controls (Cervantes et al., 2006). It remains to be demonstrated if feast-and-famine provided by SBR operation of MaB-flocs results in an enhanced phosphorous removal and an increased lipid production. Nitrogen depletion for lipid accumulation is a well-known strategy applied in algae cultures. Experimental research in parallel outdoor MaB-flocs raceways is needed to confirm for which wastewaters, biomass valorisation pathways and locations, SBRs or CSTRs are the best option. Nevertheless, outdoor MaB-floc reactor operation will always be sequential, since this system is powered by diurnal provision of sunlight.

Pond area is a major issue in MaB-S-WWT. With a HRT of 4 days (as applied in the outdoor raceway; **Chapter 7**) and pond depth of 0.4 m, a pond area of 1 ha is needed to treat 1,000 m^3 wastewater day^{-1}. For common facilities treating 500,000-10,000,000 m^3 wastewater day^{-1} (Tchobanoglous et al., 2003), treatment in MaB-flocs ponds is not realistic, particularly in Belgium. Hence it is crucial to further optimize the wastewater treatment process by determining the optimal nutrient loading rate, MaB-floc density and harvesting frequency, pond depth, temperature, timing of influent addition and mixing velocity. According to Collet et al. (2011) the main energy consumption in microalgae production is due to paddlewheel stirring, followed

by continuous pumping between pond and settler. MaB-flocs SBRs are only stirred for a very short period during night. Pumping between pond and settler is only needed for short periods during harvesting. Besides, by pumping settled MaB-flocs from the bottom of reactor, the pumping time during harvesting can be decreased. The main energy consumption in the MaB-floc raceway pond originates from the propeller pumps for reactor stirring. This was about 4 kWh or 0.80 € m^{-3} wastewater (in case of 0.15 € kWh^{-1}), whereas the total cost of CAS systems are only about 0.3-0.6 € m^{-3} wastewater (Verstraete et al., 2009). Propeller pumps were chosen above a paddle wheel to obtain a more powerful mixing of flocs, to avoid the problem of torsion of frosted paddlewheels and to decrease MaB-floc shading. Optimising the energy-efficiency of MaB-floc reactor stirring is a critical research need.

Outdoor operation of MaB-floc SBRs means dependency on weather conditions. Since oxygen production is light dependent, extra buffer tanks might be needed for wastewater storage during night time and during periods of decreased sunlight irradiation. During periods of increased BOD_5 loading, the exceptional use of mechanical aeration should be considered. The needed air blowers provide an extra cost, but can also be used for flue gas sparging during periods with increased sunlight irradiation. As temperature strongly influences biological processes, maintaining an adequate reactor temperature is a key operation factor. This can become the Achilles' heel of this technology during colder periods. The energy cost to maintain a minimum temperature of 12 °C of the MaB-floc pilot reactor during winter months was in the order of 10 € m^{-3} wastewater. This means that the availability of waste heat is of major importance for the applicability of MaB-floc SBRs in temperate climates. More research is needed in parallel raceway ponds to determine the optimal operation temperature and responses to low reactor temperature, and to screen energy-efficient systems for transfer of waste heat. Covering the pond with a plastic tunnel is another strategy. This has been recently tested in the Netherlands, but was not successful due to wind damage and overheating during summer. Considering the above challenges, the application of this technology seems especially promising for (sub)-tropical regions. Besides, international cooperation provides interesting learning environments for students and can be a stepping stone towards the valorisation of this technology as export product in line with the aimed Belgian knowledge-economy and roadmap for microalgae in Flanders (FISCH, 2013).

Table 9.1. *Advantages, challenges and future research needs of MaB-flocs SBRs regarding bioflocculation and reactor operation*

Feature	Advantages (+) or challenges (-)	⟹ Future research needs (priorities in bold)
Bioflocculation	+ Inoculum is for free and widely available	
	+ Simple and fast method to obtain adequate settling flocs	Confirm lab-scale results outdoor for each wastewater type and climate
	+ Feasibility shown on lab-scale reactors and in outdoor raceway pond	
	+ No development and maintenance of axenic monoculture needed	
	+ Monoculture not aimed at, so microalgae contamination not a problem	Test if higher resilience and increased removal of diverse community
	+ Effluent discharge possible without extra harvesting technology	
	+ No effluent contamination by flocculants	
	+ Harvesting: first concentrating step for free	
	+ Dewatering possible with large pore filter press (~200 µm)	
	- Bioflocculation mechanisms underexplored	Perform fundamental and applied research on bioflocculation mechanisms
	- Decreased OC:IC can lead to bulking sludge	
	- Black box community makes rational optimisation difficult	**Study who is doing what with whom, when and where?**
	- Floc architecture and zone dynamics (aerobic, anoxic, anaerobic)	Study the effect of floc architecture and zoning on reactor performance
	- Difficult to determine algae:bacteria ratio and community structure	**Determine community and architecture by 'Omics' and microscopy**
	- Effect of bioflocculation on reactor performance not known	Test if bioflocculation affects floc productivity and nutrient removal
Reactor operation	**+** SBR: selecting for fast settling MaB-flocs	Test if SBR operation leads to enhanced uptake of P and production of lipids
	+ SBR: flocs settling in reactor, so no need for large settling tank	Evaluate effect of duration of the settling period on the reactor performance
	+ SBR: less pumping compared to CSTR+ST	
	+ Feasible to start-up during harsh winter conditions if heated pond	
	+ Robust and stable reactor operation (during first outdoor trial)	
	- High cost of reactor stirring during day	**Improve the energy-efficiency of reactor stirring**
	- SBR: higher level of sophistication compared to CSTR+ST	**Evaluate MaB-floc SBR and CSTR+ST outdoors on various wastewaters**
	- Large pond areas are needed	**Decrease the HRT by increasing nutrient removal rates**
	- Dependency on outdoor light and temperature conditions	**Screen the MaB-floc technology in (sub-)tropical countries**
	- Heating needed during cold periods	**Develop a system for efficient use of waste heat in raceway during winter**

9.2.3. Wastewater treatment: promising, but improvement needed

The composition of different wastewaters largely varies. Although this work only screened 9 wastewater types for treatment in MaB-flocs SBRs, some key wastewater characteristics affecting MaB-floc SBRs were found. First, the IC:OC ratio of wastewaters should be balanced to avoid bulking of MaB-flocs (**Chapter 2 and 6**). Second, if inorganic carbon is lacking, flue gas can be added without affecting the MaB-floc settling (Chapter 3). However, it should be realised that this represents an extra cost that is not offset by CO_2 credits. Third, calcium-rich wastewaters are a niche opportunity for MaB-floc SBRs. Biological-influenced $CaCO_3$ precipitation in MaB-flocs improves floc settling while providing an extra function of interest to the industry: calcium removal. Extracellular carbonic anhydrase (CA) enhanced this calcium removal. This is of importance for further optimisation as wastewaters may contain compounds which inhibit CA activity, such as amides and H_2S (Tchobanoglous et al., 2003), and pharmaceutical compounds (e.g., acetazolamide is a diuretic medicine). Fourth, the C:N:P ratio of wastewater should be as such that none of these elements are limiting the overall nutrient removal needed to obtain the aimed effluent quality. For example, in manure treatment wastewater, P removal was limiting N removal (**Chapter 6**). Therefore, assessing the effectiveness and cost of nutrient addition merits further investigation. This study showed that the range of this C:N:P ratio is much wider than the Redfield molar ratio of 106:16:1. This is because microalgal biomass growth is not the only process involved in nutrient removal in MaB-S-WWT, as reviewed in Chapter 1. This was confirmed in lab-scale and outdoor SBRs (**Chapter 3, 5, 6 and 7**). Fifth, experimental screening of a wide consortium of microorganisms to develop MaB-flocs is a must, particularly if wastewaters contain toxic or dark colored compounds. Pretreatment of dark-colored wastewaters by charcoal adsorption followed by chemical precipitation leads to efficient colour removal which enables subsequent microalgal growth, but high amounts of flocculants are needed (10 g CaO L^{-1} and 0.2 g $FeCl_3$ L^{-1}) (Zamalloa, 2012).

The technical feasibility of photosynthetic aeration of aquaculture wastewater in an outdoor MaB-floc reactor in Northwest Europe was demonstrated in this work (**Chapter 7**). In fact, supersaturated oxygen levels were obtained. This can lead to inhibition of bacteria and microalgal growth (Richmond, 2004). It should be verified if lowering these oxygen levels would change the biomass growth and

biomass characteristics. In this regard, the operation of the MaB-floc raceway pond in 2014 at a food-processing company to treat COD-rich effluent, will be an interesting study.

The removal rates of carbon, nitrogen and phosphorous in MaB-floc SBRs are comparable to those of other microalgae systems, but lower than CAS systems, especially per land area (**Chapter 2, 3, 5, 6 en 7**). Understanding all the nutrient removal processes involved in MaB-floc SBRs can provide a way to enhance this removal and to promote processes towards nutrient recovery. The dependency on difficult to predict weather conditions makes rational prediction of effluent quality via modelling even more challenging.

The main challenge in the MaB-floc raceway pond treating aquaculture wastewater was N removal (**Chapter 7**). The question rises whether N removal can be enhanced by the addition of wastewater at the end of the light period to stimulate denitrification. This is not in line with the current trend towards nutrient recovery, but could be a cheap back-up system during periods of high N loading rates. Other back-up systems for N removal are nitrate resins and zeolites. Nitrate resins cost 0.5 € per g NO_3-N removed and surfactant modified zeolites cost around 0.2 € per g NO_3-N removed (Zamalloa, 2012). This means, in case of the aquaculture effluent of the raceway, over 2 € m^{-3} raceway effluent and thus not economically interesting. Another N problem in the MaB-floc raceway pond was the low nitrite removal (**Chapter 7**). The reason for this was not evidenced. In general, bacterial nitrite oxidation is inhibited by increased temperatures (30-40 °C), decreased dissolved oxygen concentrations (0.2-0.5 mg DO L^{-1}) and increased NH_3 concentrations (0.5-2 mg N-NH_3 L^{-1}) (Cervantes et al., 2006). No evidence of one of these conditions was found by following up the influent and effluent during pilot operation. Intensive monitoring of N-species during SBR cycles, could prove whether one of these conditions were present. Another hypothesis is inhibition of activity of aerobic heterotrophic bacteria by a pH above the optimum 8.3 (Park and Craggs, 2010; Oswald, 1988). Other hypotheses to explore are inhibition of nitrite oxidizing bacteria by light, $CaCO_3$ toxicity or antibiotics produced by microalgae. As pikeperches are moderately sensitive towards nitrite, acceptable levels in culturing tanks should not exceed 1.75 mg NO_2^--N L^{-1} to avoid methemoglobin formation (Wuertz et al., 2013). If recirculation of MaB-floc reactor effluent is aimed at, lowering the nitrite

concentration of effluent is crucial. To be explored remedies are prevention of nitrite formation in the influent buffer tank and enhancement of nitrite removal in the raceway or in an additional reactor via denitrification or anammox processes.

Indoor lab-scale reactor conditions strongly differ from outdoor reactor conditions. This work showed that indoor reactor performances could not be simply extrapolated to outdoor reactor performances (**Chapter 7**). The nutrient removal efficiencies were decreased by a factor 1-3. This conversion factor needs to be determined for each type of wastewater, climate and reactor operation settings.

The low phosphorous concentrations of the effluents (**Chapter 7**) show the large potential of MaB-floc SBRs for P scavenging. Poor P removal is a major barrier to the future uptake of waste stabilization pond technologies, particularly in western countries (Shilton, 2005). Therefore, MaB-floc SBRs could provide an alternative to waste stabilization ponds. As we look out to a phosphorous scarce future, both P polishing as P recovery is of strong interest. To optimize the nutrient scavenging in MaB-floc SBRs, a detailed study of nutrient recovery should be part of future work.

Increasing the functionalities of MaB-floc SBRs should benefit its implementation. Especially niches where conventional wastewater treatment technologies don't give the targeted results should be explored. A first niche opportunity is the removal of specific compounds which are difficult to remove in conventional systems, such as micropollutants including pharmaceuticals and personal care products, and color compounds including humic acids (Coriminas et al., 2013). A current problem in the chemical industry is removal of inorganic salts from their wastewater. The precipitation of minerals in MaB-flocs treating aquaculture, manure effluent, chemical industry wastewater and paper mill UASB effluent, warrants the screening of removal of various salts by MaB-flocs. A third niche could be H_2S removal. Since the growth of certain microalgae is inhibited by low concentrations of 1 mg L^{-1} H_2S (Shilton, 2005), the co-culture of photosynthetic sulfur bacteria with H_2S sensitive microalgae could induce bioflocculation.

A fourth niche is disinfection, i.e. the removal of pathogenic microorganisms including bacteria, viruses, protozoan parasites and worm parasites. Factors that cause or influence disinfection in waste stabilization ponds are expected to affect disinfection in MaB-floc reactors. These are temperature, hydraulic retention time, algal toxins, pathogen grazing by predators, sunlight disinfection, humic substances

as photosensitizers, increased pH and oxygen levels (Shilton, 2005). A preliminary screening of the microbial quality of the influent and effluent of the MaB-floc raceway treating aquaculture wastewater show that a log 2-3 removal of CFU (colony forming units) can be obtained. Nevertheless, the presence of *Enterococcus* sp. in this effluent restricts the use of this effluent as drinking water for pigs, sheep, cows and chickens (data not shown in this work). In aquaculture in Belgium, there are no legislation restrictions on the quality of water used in aquaculture tanks. If a recycling aquaculture system is aimed at in the studied pikeperch culture site, the effluent will be added to the indoor bioreactor. Hereafter it is disinfected by UV before entering the fish tanks. In this way, the microbial quality of the effluent does not restrict the recirculation of MaB-floc effluent.

Another important research need is to test the resilience and robustness of the MaB-floc SBRs for various wastewaters in various climates. In this regards, early warning systems for reactor malfunction should be developed. These could include; presence of pheophytin*a*, floc settling, odour and indicator species. Furthermore, remedies for process recovery should be developed.

9.2.4. Flue gas sparging: necessity and opportunity

In the outdoor raceway pond, flue gas addition was needed for pH control (**Chapter 7**). For several wastewaters, such as sewage, flue gas addition can optimize the C:N ratio of wastewater (**Chapter 3**). The flue gas loading rates should be balanced to avoid an effluent pH below the target norm (**Chapter 3)**. Both in lab-scale reactors as in the outdoor raceway pond, low flue gas loading rates (FGLR) were sufficient. This is of importance because a decreased FGLR means (1) decreased gas pumping and thus decreased energy and maintenance costs, and (2) decreased amount of flue gas blower systems in the raceway pond and thus decreased costs.

The often copied statement ‘microalgae can be used for flue gas scrubbing and render CO_2 credits’ should be nuanced. Indeed, in countries such as in Belgium the sparging of flue gas in open ponds does not deliver CO_2 credits (**Chapter 4**). In case flue gas is sparged in a closed photobioreactor and the offgas is emitted at a specific point, CO_2 credits may be obtained. Nevertheless, in case of sewage, this will be lower than 0.5 % of the current cost for sewage treatment of 0.5 € m^3 (**Chapter 3**). Besides, current European legislation makes off gas norms more severe due to

photosynthetic oxygen production (**Chapter 4**). This disserves further attention from policy-makers if supporting microalgae photobioreactors fed with untreated flue gas is the aim. Not only should we all be realistic on these trade revenues for flue gas compounds, but also on the flue gas volume which can be treated compared to the volume produced on an industrial site. For example, a 50 % reduction of the CO_2 content of flue gas from a coal power plant would need a total pond area of square kilometers and a huge additional network of pipelines. This is an unrealistic scenario, particularly in Belgium. The extra polishing in the algae reactors of flue gas compounds which are hazardous to the environment, is an added-value but of low importance for the industry due to the low flue gas flow rates applied compared to the total flue gas emission at an industrial plant.

Next to CO_2, flue gas contains several other compounds which can affect microalgae (**Chapter 4**). To better steer and engineer flue gas fed microalgal reactors, all these compounds need to be considered. Even treated flue gas still contains levels of compounds such as NO_x and SO_x which can be toxic for microalgae. Further research in closing nutrient balances should take into account both negative as positive effects of microalga on the removal of flue gas compounds. Based on a literature review (**Chapter 4**), this work envisaged innovative biotechnological opportunities. These include enzyme production for example carbonic anhydrase and iron siderophores for CO_2 and NO_x scavenging, enhancement of biogas and biofuel production (CH_4, H_2, alkanes) and microbial influenced mineralization of C and S compounds of flue gas. Cell wall lysis before biomass conversion by high loadings of toxic flue gas compounds, and cultivation of acidophilic species by high loading rates of flue gas are other interesting pathways.

9.2.5. Biomass harvesting: a great success story!

An efficient and cost-effective biomass harvesting is a key factor for a sustainable wastewater treatment by microalgae (Udom et al., 2013; Park et al., 2011). A major strength of MaB-floc SBRs is the easy and efficient removal of biomass from the treated wastewater without expensive biomass removal technologies and secondary contamination by chemicals. In this study, harvesting of MaB-flocs proceeded in two steps: (1) concentrating by gravity settling in a settling tank, and (2) dewatering of the settled biomass by filtering.

Table 9.2. *Advantages, challenges and future research needs and opportunities of MaB-flocs SBRs regarding wastewater and flue gas*

Feature	Advantages (+) or challenges (-) ⟹	Future research needs (priorities in bold)
Wastewater treatment	+ Mechanical aeration is not needed	
	+ Conversion of nutrients in MaB and discharge of biomass-free effluent	
	+ C:N ratio of wastewater can be enhanced by flue gas	
	+ C:N:P ratio requirements of wastewater is not as strict as Redfield ratio	
	+ Calcium removal as $CaCO_3$ is extra added value	
	+ Promising results in lab-scale reactors and outdoor raceway	
	+ Discharge norms are feasible outdoors for COD, BOD and TN and TP	
	+ - Effect of IC:OC ratio of wastewater on reactor performance	
	- Discharge norms for nitrite not reached in outdoor raceway	**Enhance nitrite removal**
	- Low nutrient removal rates per reactor area	Enhance nutrient removal rates by nutrient addition and T increase ?
	- Calcium removal decreases by CA inhibitors	Evaluate inhibition of extracellular CA by wastewater compounds
	- Modelling can not only be based on Redfield C:N:P ratio of microalgae	
	- Low understanding of nutrient removal mechanisms	**Study in detail nutrient removal mechanisms in real wastewaters**
	- No data on removal of micropollutant, pathogens, colour, salts	Evaluate the removal of micropollutants, pathogens, colour, salts
	- No data on effluent quality regarding pathogens	**Evaluate the microbial effluent quality**
	- No data on greenhouse gas emissions from outdoor open pond	**Assess the greenhouse gas emissions in an outdoor raceway pond**
	- No data on nutrient recovery	**Assess the recovery by closed C, N, P, S balances**
	- No data on optimal biomass density for each climate and wastewater	**Assess the optimal biomass density for each climate and wastewater**
	- Effect of grazers on wastewater treatment	**Assess the effect of grazers on treatment and develop control systems**
	- Early warning systems for process failure underexplored	Develop early warning systems for process failure and remedies
Flue gas addition	+ Low flue gas loading rates decrease effluent pH to discharge norm	
	+ Low flue gas loading rates don't jeopardize bioflocculation	
	+ Extra polishing of treated flue gas in open pond is an added value	
	- Flue gas addition is an extra cost for pipelines, pumping and spargers	Find or develop efficient flue gas injection systems
	- Not always CO_2, NO_x, SO_x trade revenues for flue gas in open ponds	Explore other biotechnological opportunities with flue gas & algae
	- Little info about flue gas compounds beyond CO_2 & algae	Screen the effect of all flue gas compounds on various species and interactions

For cost-effective harvesting of MaB-flocs on industrial scale, it is important that floc settling is fast and that it leads to high MaB-floc densities to decreases the pumping and settling tank dimensions. Both in lab-scale as in pilot-scale experiments treating real wastewater, MaB-flocs settled fast (**Chapter 3, 5, 6 and 7**). Up-scaling to outdoor raceway pond enhanced the MaB-flocs settling; settled MaB-flocs reached densities of around 70 g TSS L^{-1} and 20 g TSS L^{-1} (**Chapter 7**). Settling was the harvesting step with the highest MaB-floc loss, but the MaB-floc losses were still in the same range as compared to microalgae settling by flocculant addition (Udom et al., 2013) and by bioflocculation in a biomass recycling raceway (Park et al., 2011a). In this study, the remaining biomass in the supernatant was actually not lost, because it was pumped back in the pond to settle for longer periods during night.

Dewatering by filtering at 200 µm of MaB-floc biomass produced during lab-scale experiments led to a high biomass recovery. This successful dewatering was even enhanced for MaB-flocs of the outdoor raceway pond. Dewatering by this filtering led to a MaB-floc cake of 20-50 % total solids (**Chapter 5, 6 and 7**). On industrial scale, an electricity-consuming belt filter press can be used. This system has a low working cost of 0.04-0.07 € kg^{-1} dry algae (Udom et al., 2013). Also from LCA viewpoint, a belt filter press is the best option if reaching 10-90 % solids content is aimed at (Udom et al. 2013). These promising results and low working cost of filtering at 200 µm warrant further optimisation during up-scaling in industrial filter presses to find the optimal press with respect to energy-efficiency, cost-effectiveness and LCA. Outdoor operation can change the MaB-floc architecture (**Chapter 7**). This floc architecture affects the feasibility of dewatering by filter press (**Chapter 6)**. Therefore, it is crucial to determine for which wastewater types this filtering of MaB-flocs is successful.

9.2.6. Biomass productivity and valorisation: the best has yet to come

As wastewater treatment is the primary goal, a high microalgal biomass productivity is not the main driver, because the biomass is considered to be a by-product. Nevertheless, biomass valorisation is of importance for the economic feasibility of MaB-floc technology. To my opinion, a strong interest in both wastewater treatment and biomass valorisation by an industrial partner, will be a necessity for the first large-scale implementation of this MaB-floc SBR technology.

Table 9.3. *Advantages, challenges and future research needs and opportunities of MaB-flocs SBRs regarding biomass, economics, ecology, policy and legislation*

Feature	Advantages (+) or challenges (-)	⟹ Future research needs and opportunities (priorities in bold)
Biomass harvesting	+ Settling step for free to 70 g TSS L^{-1} without chemical contamination	
	+ Dewatering by 200 µm filtering results in high biomass recovery	
	+ Up to 50 %TS of algal cake after hydropress filtering at 200 µm	
	- So far only results of pilot scale and not of industrial scale filter press	**Find the most energy-efficient & cost-effective industrial filter press**
	- Harvesting is affected by MaB-floc architecture and wastewater type	**Screen filtering with outdoor MaB-flocs fed with various wastewaters**
Biomass productivity and valorisation	+ Potential as shrimp feed: small amounts of MaB increase shrimp colour	**Test larger amounts of MaB-flocs in diets for various aquaculture sp.**
	+- Strong decrease in biomass productivity during up-scaling	Increase biomass productivity if interesting valorisation pathway is found
	+ Dominance of one microalgal species in first outdoor MaB-floc raceway	
	- Biogas potential of MaB-flocs is low	Screen pretreatment of MaB-flocs to increase biogas production
	- Underexplored biomass valorisation pathways for various wastewaters	**Screen various pathways for biomass valorisation**
	- Species dominance remains to be shown for various wastewaters	Find ways to steer MaB-floc community structure
Economics and ecology	+ No need for expensive mechanical aeration of wastewater treatment	
	+ Relatively cheap harvesting by gravity settling and filtering at 200 µm	
	- LCA and exergy evaluation is lacking (will be done by EnAlgae partner)	**Improve the sustainability by LCA and exergy analysis**
	- Economic evaluation is lacking (will be done by EnAlgae partner)	**Evaluate and optimize the economics**
Policy and legislation	+ MaB-floc SBRs technology fits in programs for innovative clean-tech	
	+ Limited expertise abroad gives opportunities for technology export	**Evaluate MaB-floc SBRs in areas with abundant light, heat and land**
	- Wastewater treatment not a priority in Flemish algae roadmap	Highlight benefits of MaB-floc SBRs versus microalgal technology
	- Legislation restrictions for aquaculture and manure fed MaB-flocs	**Evaluate microbial quality of MaB-flocs & suggest legislation adjustment**
	- No standard methods for CO_2, NO_x, SO_x trade revenues in algae reactors	
	- More severe off-gas emission norms regarding O_2 reference method	Test other reference methods for off-gas and discuss with policy-makers

During lab-scale experiments, extreme high biomass productivities were obtained (e.g., 160 ton TSS ha^{-1} year for aquaculture wastewater; **Chapter 6**). This study showed that up-scaling to an outdoor raceway pond decreased the MaB-floc biomass productivity to 33 ton TSS or 12 ton VSS ha^{-1} pond area $year^{-1}$ (**Chapter 7**). This biomass productivity is low compared to other studies on microalgae production combined with wastewater treatment. One of the reasons was biomass loss due to predators. The presence of *Tubifex* sp. in the biomass can be beneficial for valorisation as aquaculture feed ingredient, since *Tubifex sp*. have a higher nutritional value (animal protein) and can increase appetites and palatability of fish (Lietz, 1988). In this way, MaB-flocs may provide an interesting ingredient for aquaculture feed. Once a sustainable and lucrative pathway of biomass valorisation is demonstrated on a larger scale, an increased biomass productivity should be aimed at.

Scale-up from indoor reactors to an outdoor raceway pond led to a drastic shift in the community structure, increased the microalgae, ash and chlorophyll content of the MaB-flocs and enhanced the floc settling (**Chapter 7**). These changes highlight the importance of outdoor studies.

It does not need many difficult calculations to realize that conversion of microalgae to methane containing biogas will not 'save the combustion machine' nor catalyze the implementation of MaB-floc technology on the short term. Even after pretreatment of MaB-flocs by flue gas sparging, microwave heating, freezing, acid addition and co-digestion with fish sludge, a biogas production of 0.13-0.30 Nm^3 kg^{-1} volatile solids (VS) was obtained (data not shown of this ongoing research). The current value of biogas is about 0.2 € Nm^{-3}. This means 0.03-0.06 € per kg MaB-floc. In general the low digestibility of microalgae is due to the difficulty to break the microalgae cell wall and due to ammonia inhibition (Zamalloa, 2012). Besides, extreme $CaCO_3$ contents of MaB-flocs could lead to problematic scaling of digesters and pipelines.

To screen whether MaB-floc biomass can be valorised for higher-value products such as aquaculture feed, outdoor MaB-flocs were added to the diets of Pacific white shrimp (*Litopenaeus vannamei* L.). This showed that a low inclusion of 8 % of MaB-flocs in the shrimp diets did not alter the shrimp productivity, while the colour of cooked shrimps was enhanced (**Chapter 8**). Further research should screen an enhanced inclusion of MaB-flocs in diets of shrimps and other aquaculture species,

especially with regard to the high ash content of MaB-flocs. For aquaculture applications of MaB-floc biomass, it is crucial to screen for pathogens which can grow on microalgal products and to find beneficial-associated bacteria that can outcompete pathogens in microalgal flocs (Natrah et al., 2013). An important opportunity is the production of antimicrobials by certain algae which inhibit the growth of aquaculture pathogens such as *Staphylococcus* sp. and *Vibrio* sp. (Natrah et al., 2013).

Compounds of interest for the broad chemical industry are functional molecules, monomers and biomass as filler material (FISCH, 2013). The production of pharmaceutical products such as anti-cancer products present in certain microalgae, will be restricted to MaB-floc production on specific wastewaters and is a long-term research and development process. The use of MaB-flocs as biofertilizers and plant growth enhancer for horticulture is another pathway which is currently being investigated. Due to the presence of $CaCO_3$ in MaB-flocs, a mineral used in paper production, it seems worthwhile to investigate if MaB-flocs can be used as material for paper. An innovative combination of paper and fertilizer could be the MaB-floc sheet: a 'lasagna' of paper, MaB-flocs, water crystals and seeds to be used in gardens. A reduced price and steady supply of biomass, and a high and stable concentration the target compounds from the MaB-flocs biomass, are of major importance. With this regard, MaB-floc production in less varying conditions of tropical climates might be beneficial.

9.2.7. Economics and ecology: important research for 2014

Promising economics are a necessity for a successful implementation of MaB-floc technology, but was not the subject of this study. A detailed economic study of MaB-floc SBRs treating aquaculture wastewater will be performed within the EnAlgae project (drs. E. Vulsteke & prof. J. Albrecht, Ghent University) and will be based on the outdoor raceway pond results of this work. To make an accurate assessment, certain pilot materials should be changed by industrial-scale materials. The quest of a low-energy consuming stirring system is of major importance. A potential lies in the valorisation of microalgal biomass, especially because a strong dominance of one microalgal species in the outdoor MaB-flocs was obtained (**Chapter 7**). The current market prices for microalgae range from 8 € to 400 € kg^{-1} dry matter (FISCH, 2013). A price of 8 € kg^{-1} MaB-flocs would result in 96,000 € ha^{-}

[1] pond area year^{-1} or 0.26 € m^{-3} wastewater. Taking into account the current cost of wastewater treatment of 0.30-0.60 € m^{-3}, the first predictions show that there is economic potential that merits a detailed analyses and further optimisation.

To improve the sustainability of MaB-floc SBR technology, LCA and exergy analyses will be performed within the EnAlgae project (drs. S.E. Taelman, drs. S. Sfez & prof. Dewulf, Ghent University). Measurements of greenhouse gas emissions from the MaB-floc raceway pond will be needed to make an accurate assessment and comparison with conventional activated sludge systems for wastewater treatment.

9.2.8. Legislation and policy: positive and negative catalyzers

Themes regarding legislation on microalgae and wastewater treatment and flue gas injection that need to be clarified by local policy makers are various. The type of reactor (open or closed) that is allowed for flue gas treatment to emission norms and its concomitant off-gas discharge standards in different countries need to be clarified. A standard method to determine the amount of earned CO_2, NO_x and SO_x credits from flue gas injection in algae rectors needs to be set (**Chapter 4**). The adjustment of the O_2 reference method for flue gas treatment in microalgae reactors is needed, since the oxygen that is being produced in these reactors make current emission norms more difficult to obtain compared to oxygen using or oxygen neutral systems (**Chapter 4**). As depicted in **Chapter 8**, the valorisation as feed ingredient of MaB-flocs fed with wastewater containing manure particles is restricted by current European legislation, but not for food-processing industry wastewater. Therefore opportunities lie in MaB-floc treatment of this wastewater type. In this regard, the operation in 2014 of the MaB-floc raceway pond at a food-processing company to treat UASB effluent will be an interesting case-study.

In the rapport 'a roadmap for microalgae in Flanders', managed by Flanders Innovation Hub for Sustainable Chemistry, wastewater treatment by microalgae is not mentioned as a priority research topic (FISCH, 2013). Nevertheless, MaB-floc biomass could provide a chemical industry feedstock which is cheaper compared to non-flocculating microalgae grown on synthetic media. Moreover, the EnAlgae Pilot Calls showed a large interest of Flemish companies from the chemical and food-processing sector. Next to research, effective communication and close involvement of all stakeholders is important in the further development of this technology.

Innovation in clean-tech products is part of the new Flemish industry policy and is being promoted as Flemish export product. Further Flemish funding to perform research regarding MaB-floc technology should thus be searched in clean-tech programs and private investors.

9.3. Future perspectives: applications of MaB-floc SBRs

The application of MaB-floc SBR technology requires some specific conditions: suitable wastewater, available land area, sunlight, heat and a feasible biomass valorisation pathway (Table 9.4.). Moreover, this technology will only be implemented where resources are available for new investments and where legislation does not restrict its implementation. Furthermore, this technology will only be applied in specific circumstances where it presents a high added value, i.e. an improvement compared to the conventional technology. This added value can be addressing an economical problem, such as lowering the cost:benefit ratio, and/or addressing an ecological problem, such as reaching stringent effluent discharge norms, lowering greenhouse gas emissions and increasing nutrient recovery.

The above listed requirements were very generally projected on various important Belgian industries (Table 9.4.). Large potential lies in the treatment of aquaculture wastewater. However, the emerging Belgian aquaculture industry is still small. The implementation of this technology will also be restricted to areas where waste heat is available. In aquaculture, there is no large availability of waste heat during winter. Besides, current European legislation limits the valorisation of wastewater-fed MaB-floc biomass as feed ingredient.

As for the chemical and food-processing industries, outdoor experimental trials are needed to screen the technical and economic feasibility and the reliability in outdoor conditions. The question rises whether MaB-floc SBR technology will present a high enough added value for industries in Belgium. The huge land area requirement (probably up to 20 times the area of a conventional activated sludge system) could offset the advantage of cost-savings on mechanical aeration of conventional activated sludge systems. Further optimisation of the nutrient removal, energy efficiency and finding an interesting biomass valorisation pathway will be of major importance, if not crucial. Furthermore, the environmental impact and its economic value needs to be assessed based on outdoor pilot scale studies with these

specific wastewaters. Niche opportunities to address current problems in wastewater treatment such as removal of phosphorous, salts and micropollutants merit further investigation to increase the added value of MaB-floc SBRs.

To date, only a very general assessment of the potential of MaB-floc SBRs in temperate climates can be made, since, to the best of our knowledge, published research results on MaB-floc SBRs for wastewater treatment outdoor in a temperate climate are limited to this work. In 2014, UASB effluent from the food-processing industry will be treated in an outdoor MaB-floc pilot reactor. Similar work with outdoor MaB-floc reactors in continuous stirred reactors with settling tanks is currently being performed in New Zealand. A raceway pond with recycling of the harvested microalgal flocs has been treating sewage for several years (Park et al., 2012). In 2014, MaB-floc pilot reactors (named 'ALBA reactors') will be operated by the Papiertechnische Stiftung in Germany to treat paper mill wastewater and by Celabor in southern Belgium to treat food-processing wastewater. These studies are of large importance to assess the potential of MaB-floc technology in temperate climates, especially regarding its efficiency.

Considering the required heat, sunlight and land area for MaB-floc SBRs and the importance of a biomass valorisation pathway, large potential lies in the application of this technology in the aquaculture industry in (sub-)tropical areas. There is a huge need for wastewater treatment systems in certain (sub-)tropical aquaculture companies, for example for shrimp farmers. Especially integrated systems are gaining more attention. Furthermore, these shrimp farmers already have a strong expertise in algae and pond management. In a first step, the proof-of-principle of treatment of aquaculture effluent combined with biomass valorisation of fresh MaB-flocs as diet ingredient for shrimp needs confirmation for brackisch and saltwater. Secondly, further optimisation outdoors of the operational parameters (hydraulic retention time, MaB-floc retention time, harvesting frequency, MaB-floc density, flue gas addition for pH control, reactor stirring) will be needed to increase the nutrient removal and energy efficiency and to enhance the MaB-floc composition in terms of feed quality. Moreover, the economic potential and the impact on the environment, animal health and food security of MaB-floc SBRs should be carefully assessed.

***Table 9.4**. General requirements for and potential added value of MaB-floc SBR technology applied in various industries*

Characteristics	**Belgium**			**(Sub-)tropical countries**
	Food-processing industry	**Chemical industry**	**Aquaculture**	**Aquaculture**
Requirements				
Suitable wastewater	Only lab results	Only lab results	Pilot results	No results yet
Land area	Can be an issue	Can be an issue	Can be an issue	No strong issue
Heat (ambient or waste)	Waste heat often present	Waste heat often present	No waste heat	No need for heating
Sunlight	Strong seasonal variations	Strong seasonal variations	Strong seasonal variations	Low seasonal variations
Biomass valorisation pathway	To be found	To be found	Feed ingredient potential	Feed ingrediend potential
Investment resources	Large industry	Large industry	Emerging industry	Large industry, increasing product prices (e.g. shrimp)
Potential added value				
Economical	Decreased aeration costs? [1] Biomass valorisation?	Decreased aeration costs? Biomass valorisation?	Decreased aeration costs Biomass valorisation?	Decreased aeration costs? Decreased feed costs? Effluent reuse?
Ecological	Improved effluent quality?	Improved effluent quality?	Improved effluent quality?	Strongly improved effluent quality?
Further research	Outdoor pilot needed (running) Optimisation needed Increase of added value : P	Outdoor pilot needed Increase of added value: micropollutant & EC removal	Optimisation needed: N removal and energy efficiency	Proof-of-principle lab scale Optimisation outdoors Valorisation as feed ingredient
Opportunities	Legislation feed market	Biomass valorisation expertise		Expertise in algae and ponds

[1] *?: Needs to be confirmed by scientific research in outdoor reactors.*

9.4. Overall conclusions

This work presents a novel microalgal technology for wastewater treatment based on photosynthetic aeration and bioflocculation. To address the high costs for separating the algae from treated wastewater, microalgal bacterial flocs in sequencing batch reactors (MaB-floc SBRs) were developed and screened from lab-scale to outdoor pilot-scale. Especially MaB-floc settling and dewatering by filter press with large pores was very successful. Lab-scale experiments revealed that there is potential for various wastewaters. Calcium removal and phosphorous polishing from wastewater are niche opportunities which increase the functionality of MaB-floc SBRs. Further optimisation regarding nutrient removal and recovery, energy-efficient stirring and heating, biomass valorisation pathways, and economic and environmental impact analyses are needed to set the stage for its industrial implementation.

'Deure rijen mee ui karre!'[1]

(L. Van Den Hende, 2013)

1. West-flemish expression of my father meaning 'Go on!'

One for all(gae),

and all(gae) for one.

Picture on previous page:
Part of the construction team of the MaB-floc raceway pond: Bewaren en Bebouwen nv, CATAEL bvba, Inagro and Howest (now UGent).

SUMMARY

SAMENVATTING

Summary

Sunlight-powered **microalgal bacterial systems for wastewater treatment** (MaB-S-WWT) are based on photosynthetic aeration which replaces the costly mechanical aeration of conventional activated sludge systems. Moreover, microalgae scavenge nutrients from the wastewater, in line with the requested paradigm shift towards nutrient-recovering wastewater treatment plants. However, the amount of publications on these microalgal systems for wastewater treatment largely outnumbers the amount of their industrial applications, especially in Northwest Europe. As reviewed in **Chapter 1**, **several challenges hamper the implementation** of MaB-S-WWT. This dissertation addressed some of these challenges. A major hurdle in MaB-S-WWT is the separation of the microalgal bacterial biomass from the treated wastewater. Therefore, in this dissertation a novel concept is presented**: microalgal bacterial flocs in sequencing batch reactors (MaB-floc SBRs**) for wastewater treatment. MaB-flocs are easily and fast developed via bioflocculation of a consortium of local microalgae and bacteria. MaB-flocs settle quickly by gravity. After a settling period in the reactor, biomass-free effluent can be withdrawn. Hereafter influent is added again and treated by MaB-flocs.

Wastewaters largely differ in their inorganic carbon: organic carbon (IC:OC) ratio. In **Chapter 2**, it is shown that MaB-floc properties are improved by a balanced **IC:OC ratio**. An increased IC:OC ratio decreases the MaB-floc settling by the dominance of filamentous microalgae in the floc and decreases the nutrient removal from the wastewater. This implies that MaB-floc SBRs seem more suitable for secondary treatment of wastewater compared to tertiary treatment.

MaB-flocs for secondary treatment of sewage entails several challenges. The carbon:nitrogen ratio (C:N) is too low to scavenge all nitrogen from sewage. Moreover, microalgal photosynthesis increases the reactor pH to values above the discharge norm and microbial optimum. A pH above 9.3 leads to nitrogen loss via ammonia volatilization. Flue gas supplementation may provide a solution to all these challenges. However, the **flue gas flow rate** (FGFR) has to be limited to avoid algal toxicity and to reach stringent off gas and effluent limits. Therefore, the potential of MaB-floc SBRs was investigated for the **secondary treatment of sewage** sparged with different FGFR (**Chapter 3**). Effluent (N, P and pH) and off gas discharge levels (NO_x, SO_x) met the European discharge limits with a hydraulic retention time of only

0.7 days and a FGFR of 0.6 L h^{-1}. This low FGFR provided sufficient carbon and resulted in removal efficiencies of 48 ± 7 % CO_2, 87 ± 5 % NO_x and 99 ± 1 % SO_2. MaB-flocs settled fast reaching up to a density of 19 g volatile suspended solids (VSS) L^{-1}. High biomass productivities (0.18 VSS g L^{-1} day^{-1}) were obtained under a low light intensity. This successful reactor performance indicates the large potential of MaB-flocs for flue gas sparged sewage treatment.

Next to CO_2, **flue gas** contains several other **compounds** which can positively or negatively interact with microalgae. To better steer and engineer flue gas fed microalgae reactors, all these compounds need to be considered. Therefore, the chemical composition of flue gas, current flue gas treatment technologies, and biochemical interactions of flue gas compounds and microalgae are reviewed in **Chapter 4**. Novel pathways for valorisation of microalgae and flue gas compounds are envisaged. These include enzyme production for environmental technology, novel biogas production and biosequestration of minerals. Furthermore, fundamental and applied research niches that merit further investigation are highlighted.

Calcium-rich wastewater from recycling paper mills results in problematic scale formation. In **Chapter 5** it is shown that MaB-floc SBRs significantly **remove calcium** from this effluent **via biomineralisation to $CaCO_3$.** Inhibiting extracellular carbonic anhydrase (CA), an enzyme catalysing the hydradation/dehydradation of CO_2, significantly decreases the removal of calcium and inorganic carbon in MaB-floc SBRs. This implies that CA-inhibitors present in certain wastewaters can decrease calcium removal in these reactors. MaB-flocs contained 10 ± 3 % calcium and biologically-influenced calcite crystals. Harvesting by filtering at 200 µm recovered over 99 % of the MaB-flocs. This successful harvesting addresses a major bottleneck of microalgae wastewater treatment. This proof-of-principle warrants further improvement towards an increased calcium removal.

Since the composition of (agro-)industrial wastewaters largely varies, research efforts should be undertaken to effectively pinpoint the valorisation potential of MaB-floc SBRs and assess its industrial applicability. Therefore, MaB-floc SBRs were screened for **wastewaters from various (agro-)industries**: aquaculture, manure treatment, food-processing industry and chemical industry (**Chapter 6**). These wastewater types resulted in significant different removal rates of organic carbon, inorganic carbon, nitrogen and phosphorous parameters. The low phosphorous

concentrations in manure and aquaculture effluent (0.19-0.40 mg total P L^{-1}) highlight the potential of MaB-floc SBRs for phosphorous polishing. This is important because phosphorous polishing is of large interest nowadays. The average biomass productivities of MaB-floc SBRs ranged from 0.14 to 0.26 g Total Suspended Solids (TSS) $L_{reactor}^{-1}$ day^{-1} with 43 to 87 % VSS/TSS. Harvesting by filtering at 200 µm recovered 79-99 % of MaB-flocs and resulted in 12-21 % total solids (TS). Although for some wastewaters further optimisation is needed to reach current discharge norms, the results of this study demonstrate the wide applicability of MaB-floc SBRs for wastewater treatment in (agro-)industries.

On (agro-)industrial scale, MaB-flocs SBRs should be operated outdoors in a raceway pond. Indoor performances of MaB-flocs SBRs cannot be simply extrapolated to performances in an outdoor raceway pond because the reactor conditions strongly differ. Therefore MaB-floc SBRs were **up-scaled from indoor photobioreactors of 4, 40 and 400 L to a 12 m^3 outdoor raceway pond** (**Chapter 7**). Up-scaling decreased the nutrient removal efficiencies with a factor up to 3 and biomass productivities with a factor 1-13. However, the effluent still met current discharge norms, except for nitrite and nitrate during winter. Flue gas sparging of the raceway pond was needed to decrease the effluent pH. Outdoor MaB-flocs showed enhanced settling properties and an increased ash content compared to indoor MaB-flocs. Flocs were efficiently harvested (99 %) by settling and dewatering by a hydropress with 150-250 µm filter to 43 %TS. Optimisation of nitrogen removal and biomass valorisation are future challenges towards industrial implementation.

MaB-floc SBRs not only treat wastewater, but also produce microalgal bacterial biomass. This biomass needs valorisation. To screen whether **MaB-floc biomass** can be **valorised as shrimp feed ingredient**, dried MaB-flocs were added to the diets of Pacific white shrimp (*Litopenaeus vannamei* Boone, 1931) (**Chapter 8**). This study shows that a low inclusion of 8 % of MaB-flocs in the diet of these shrimp did not affect the shrimp survival, weight gain and food conversion, nor did they affect the proximate composition and fatty acid profile of the raw shrimp muscle. It was demonstrated that increasing the MaB-flocs share in shrimp diets significantly increases the pigmentation (redness and yellowness) of cooked shrimp tails. This increases the market value of shrimps. Future research is needed to determine the maximum inclusion of MaB-flocs in diets of shrimp and other aquaculture species.

Chapter 9 finishes with a **general evaluation of MaB-floc SBR technology for wastewater treatment** and **future perspectives** based on the obtained results. To conclude, the main strength of the presented MaB-floc SBR technology is the easy development, the fast settling and successful harvesting of MaB-flocs. Further optimisation regarding nutrient removal, energy-efficiency, biomass valorisation pathways, economic feasibility and sustainability analyses are needed to set the stage for its industrial implementation.

Samenvatting

In **microalgenbacteriënsystemen voor afvalwaterzuivering** (MaB-S-AWZ) wordt zuurstof geproduceerd via fotosynthese op basis van zonlicht. Deze zuurstofbeluchting vervangt de dure mechanische beluchting van conventionele actief slib systemen (CAS). Daarenboven nemen microalgen nutriënten op uit het afvalwater. Dit beantwoordt de huidige vraag naar nutriëntenrecuperatie in afvalwaterzuivering. Er zijn al heel veel artikels over MaB-S-AWZ gepubliceerd. Het aantal uitvoeringen in de praktijk op industriële schaal blijft echter beperkt in Noordwest Europa. **Verschillende knelpunten verhinderen de toepassing van deze systemen in de praktijk**. Deze knelpunten worden beschreven in **Hoofdstuk 1**. Dit werk heeft tot doel oplossingen te zoeken voor verschillende van deze knelpunten. Eén van de grootste problemen is de scheiding van de microalgenbacteriënbiomassa van het gezuiverde water. Om dit probleem aan te pakken, wordt in dit werk een nieuw concept voorgesteld: **microalgenbacteriënvlokken in een sequentiële batch reactor (MaB-vlokken SBR)** voor afvalwaterzuivering. MaB-vlokken kunnen eenvoudig en snel ontwikkeld worden via bioflocculatie van een consortium van lokale microalgen en bacteriën. Deze vlokken bezinken snel. Na een bezinkingsfase, kan het gezuiverde water weggepompt worden terwijl de MaB-vlokken in de reactor blijven. Daarna wordt opnieuw afvalwater toegevoegd aan de reactor om door deze MaB-vlokken gezuiverd te worden.

De verhouding van anorganische en organische koolstof (AC:OC) in afvalwater varieert sterk. In **Hoofdstuk 2** werd aangetoond dat de eigenschappen van MaB-vlokken worden verbeterd door een evenwichtig **AC:OC verhouding**. Een verhoogde AC:OC verhouding leidt tot een minder goede bezinking als gevolg van dominantie van filamenteuze microalgen in de vlok. Dit leidt ook tot een verminderde nutriëntenverwijdering uit het afvalwater. Dit betekent dat een MaB-vlokken SBR met name geschikt is voor een secundaire behandeling van afvalwater en minder geschikt zijn voor een tertiaire behandeling.

MaB-vlokken voor de secundaire behandeling van rioolwater kent verschillende knelpunten. De koolstof:stikstof verhouding (C:N) is te hoog om alle stikstof uit het afvalwater om te zetten tot biomassa. Daarenboven verhoogt fotosynthese door microalgen de reactor pH tot waarden boven de lozingsnorm en boven het microbieel optimum. Een pH hoger dan 9,3 leidt tot stikstofverlies door

vervluchtiging van ammoniak. Rookgasinjectie kan een mogelijke oplossing bieden voor deze knelpunten. In **Hoofdstuk 3** werd daarom nagegaan wat het potentieel is van een MaB-vlokken SBR voor de secundaire **behandeling van rioolwater** in combinatie met rookgasinjectie. Het rookgasdebiet moet echter beperkt worden om toxiciteit voor microalgen te vermijden en om onder de emissienormen voor het gezuiverde rookgas te blijven. Daarom werden **verschillende rookgasdebieten** vergeleken. Het effluent (stikstof, fosfor en pH) en het afgas (NO_x, SO_x) haalde de Europese lozings- en emissienormen met een lage hydraulische verblijftijd van 0,7 dagen en een rookgasdebiet van 0,6 L h^{-1}. Dit laag rookgasdebiet gaf voldoende koolstof en resulteerde in een verwijderingsefficiëntie van 48 ± 7 % CO_2, 87 ± 5 % NO_x en 99 ± 1 % SO_2. MaB-vlokken bezonken snel tot een densiteit van 19 g vervluchtigbare zwevende stoffen (VZS) L^{-1}. Een hoge biomassa productiviteit (0.18 g VZS L^{-1} dag^{-1}) werd behaald bij een lage lichtintensiteit. Deze veelbelovende resultaten tonen aan dat er potentieel is om rioolwater te behandelen in een MaB-vlokken SBR voorzien van rookgasinjectie.

Naast CO_2 **bevat rookgas verschillende** andere **componenten** die positief of negatief kunnen interageren met microalgen. Om de sturing en engineering van microalgen reactoren met rookgasinjectie te verbeteren is het belangrijk om al deze componenten te beschouwen. In **hoofdstuk 4** wordt daarom een overzicht gegeven van de samenstelling en huidige behandelingstechnieken van rookgas en van de biochemische interacties van rookgascomponenten met microalgen. Vernieuwende mogelijkheden voor de valorisatie van microalgen en rookgas worden voorgesteld. Verder worden niches voor fundamenteel en toegepast onderzoek belicht.

Calciumrijk afvalwater van papierproductie resulteert in problematische kalkaanslag. In **hoofdstuk 5** wordt aangetoond dat een MaB-vlokken SBR **calcium verwijdert via biomineralisatie tot $CaCO_3$**. Carbonic anhydrase (CA) is een enzyme dat de hydratie/dehydratie van CO_2 katalyseert. Inhibitie van extracellulaire CA vermindert significant de verwijdering van calcium en anorganische koolstof in een MaB-vlokken SBR. Dit wil zeggen dat de aanwezigheid van CA-inhibitoren in afvalwater de calciumverwijdering in deze SBR kan verminderen. MaB-vlokken bevatten 10 % calcium waaronder als calcietkristallen. Gaasdoek met maaswijdte van 200 µm werd gebruikt om de reactorinhoud met MaB-vlokken te filteren. Zo konden 99 % van de MaB-vlokken geoogst worden. Dit succesvol oogsten is een groot

voordeel tegenover andere MaB-S-AWZ. Verder onderzoek is echter nodig om de calciumverwijdering te optimaliseren.

Omdat de samenstelling van (agro-)industrieel afvalwater sterk varieert, werd het potentieel van een MaB-vlokken SBR gescreend voor de **zuivering van afvalwater** afkomstig van **verschillende (agro-)industriële sectoren**: aquacultuur, mestverwerking, voeding en chemie (**Hoofdstuk 6**). Deze afvalwatertypes resulteerden in significant verschillende verwijderingssnelheden van organische koolstof, anorganische koolstof, stikstof en fosfor. De lage fosfor concentraties na behandeling van afvalwater van mestverwerking en aquacultuur (0.19-0.40 mg totaal P L^{-1}) benadrukt het potentieel van een MaB-vlok SBR voor verregaande fosforverwijdering. Dit is belangrijk want er is momenteel een grote vraag hiernaar. De gemiddelde biomassa productiviteit van de MaB-vlokken reactoren lag tussen 0.14 g en 0.26 g totale zwevende stoffen (TZS) $L_{reactor}^{-1}$ dag^{-1} met 43 tot 87 % VSS/TSS. Oogsten door filteren (200 µm) resulteerde in een MaB-vlokken recuperatie van 79-99 % en leidde tot een sterk ontwaterde algenkoek met 12-21 % droge stof. Niettegenstaande er voor verschillende afvalwaters verdere optimalisatie nodig is, demonstreert deze studie de brede toepasbaarheid van MaB-vlokken SBR technologie voor afvalwaterzuivering in (agro-)industriële sectoren.

Op (agro-)industriële schaal dient een MaB-vlokken SBR buiten te worden aangelegd in een renbaanvijver. De resultaten van labo-onderzoek kunnen niet eenvoudigweg geëxtrapoleerd worden naar resultaten buiten in een vijver. Reactorcondities binnen en buiten verschillen sterk. Daarom werden **MaB-vlokken SBR reactoren opgeschaald van indoor fotobioreactoren van 4 L, 40 L en 400 L tot een outdoor renbaanvijver van 12 m^3** (**Hoofdstuk 7**). Opschalen verminderde de efficiëntie van nutriëntenverwijdering en biomassaproductiviteit met een factor 1-13. Het effluent van de MaB-vlokkenvijver behaalde de beoogde lozingsnormen, behalve voor nitriet en voor nitraat tijdens de winterperiode. Rookgasinjectie was nodig om de pH van het effluent te verlagen tot de lozingsnorm. Outdoor MaB-vlokken bezonken beter en bevatten meer as in vergelijking met de indoor MaB-vlokken. De MaB-vlokken in de vijver werden geoogst in twee stappen. In een eerste stap werden ze opgeconcentreerd door bezinking. In een tweede stap werden ze ontwaterd door te filteren (~ 200 µm) tot een algenkoek met 43 % droge stof. Zo kon

99 % van de MaB-vlokken geoogst worden. Optimalisatie van de stikstofverwijdering en biomassavalorisatie moet deel uitmaken van verder onderzoek.

Een MaB-vlokken SBR behandelt niet alleen afvalwater, maar produceert ook **microalgenbacteriënbiomassa**. Deze biomassa moet gevaloriseerd worden. Om na te gaan of deze biomassa kan aangewend worden **als ingrediënt voor garnalenvoeder** werden gedroogde MaB-vlokken toegevoegd aan voeder voor Pacifische witte garnalen (*Litopenaeus vannamei* Boone, 1931) (**Hoofdstuk 8**). Deze studie toonde aan dat 8 % inclusie van MaB-vlokken in garnalenvoeder de overleving, de groei en de voederconversie van garnalen en de biochemische samenstelling van garnalenstaarten niet wijzigt. Ook werd aangetoond dat het verhogen van het aandeel van MaB-vlokken in het garnalendieet de rode en gele pigmentatie van garnalenstaarten versterkt. Dit is interessant omdat het de marktwaarde van garnalen kan verhogen. Verder onderzoek moet uitmaken wat het maximum aandeel van MaB-vlokken in voeder voor garnalen en andere aquacultuurdieren is.

Hoofdstuk 9 sluit dit werk af met een **algemene evaluatie** van MaB-vlokken SBR technologie voor afvalwaterzuivering samen met **voorstellen voor verder onderzoek**. Als algemene conclusie kan gesteld worden dat de grote sterkte van MaB-vlok SBR technologie de eenvoudige ontwikkeling, de snelle bezinking en het succesvol oogsten van MaB-vlokken is; zeker in vergelijking met andere microalgensystemen voor afvalwaterzuivering. Verdere optimalisatie is nodig met betrekking tot nutriëntenverwijdering, energie-efficiëntie, biomassavalorisatie, economische haalbaarheid en duurzaamheid, vooraleer deze technologie marktrijp is.

The amount of papers on algae largely outnumbers the amount of industrial applications of microalgal technology.

Picture on previous page:

Printscreen of search for information on ‘algae wastewater treatment’ on scholar.google.be.

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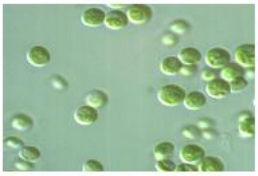
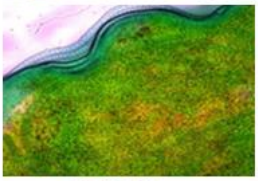
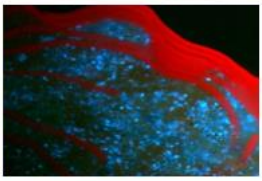
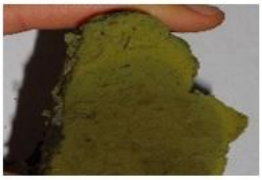







Thx ☺

Picture on previous page:

Logos and pictures of members of the ‘MaB-floc community *sensu lato’*.

ACKNOWLEDGEMENTS

DANKWOORD

Acknowledgements / Dankwoord

Hier ligt het dan, het neergeschreven eindresultaat van het uitdokteren van een ‘symbiose’ tussen de wondere wereld van de microalgen en de ingenieuze kant van milieutechnologie. Er kwam veel bij kijken, van samen concepten bedenken, reactoren in elkaar knutselen, aanbestedingen en contracten uitschrijven, afvalwater en data analyseren, events organiseren, lessen uitwerken, tot papers en hoofdstukken neerschrijven. Een enorm boeiende leerschool voor het ontdekken van een nieuwe passie, zo kan je het op zijn minst noemen. Al dat werk heb ik natuurlijk niet alleen gedaan. Ik wil iedereen dan ook heel erg bedanken, zonder jullie was het niet gelukt!

Zonder ‘economic resources’, was er geen onderzoek en geen doctoraat. Graag bedank ik Howest en UGent voor de mogelijkheid om als academisering-assistent niet alleen van het onderzoekswerk maar ook van het lesgeven te mogen proeven. Het smaakte naar meer! Ook bedank ik de Europese Gemeenschap (INTERREG IVB NWE), de Vlaamse Gemeenschap en de Provincie West-Vlaanderen voor de financiële steun van het EnAlgae project. Zonder dit project, was er geen pilootreactor. Het Ernest du Bois fonds, beheerd door de Koning Boudewijn Stichting, bedank ik voor het geloof in mijn doctoraatsonderzoek. Zonder jullie, waren er geen MaB-vlokken etende garnalen.

Ook zonder ‘human resources’, geen onderzoek en geen doctoraatsthesis. Promotoren, van harte dank voor de begeleiding tijdens dit algenavontuur over toppen en door dalen naar een eindtop! Nico, dank voor je input en frisse kijk op de experimentele set-up, resultaten, papers en reviewers feedback. Je waardevolle en snelle feedback maakten het mogelijk om de deadline van mijn schrijfrace de voorbije herfst te halen. Je harde werkkracht werkte enorm motiverend. Han, je stimuleerde me om het onderste uit de kan te halen en om heel zelfstandig te werken. Voor al mijn praktische en theoretische vragen kon ik altijd bij je terecht. Jouw waardevolle input en boeiende discussies hebben me verder laten groeien in het worden van een meer kritische onderzoeker. Jouw mantra ‘wie schrijft, die blijft’ heeft me doen schrijven ... en nog steeds. Han & Nico, de eindtop van dit doctoraatsonderzoek omtrent algen werd bereikt, maar de eindstop van algenonderzoek ligt nog heel ver in de toekomst...

Without Ph.D. Examination Committee, no Ph.D. thesis. Prof. Bossier, prof. Devlieghere, dr. Ernst, prof. Goethals, prof. Sorgeloos, dr. Steyer, thank you for taking the time to examine this work and offer me your valuable remarks and suggestions. I

enjoyed the discussions during the predefence and I will keep them in mind to improve future work on MaB-flocs.

Zonder een ondersteunend team van collega's, geen doctoraat. Hartelijk dank voor alle ondersteuning van (ex-)collega's van Howest, UGent Campus Kortrijk, Biogaslabo, Biogas-e en Vlakwa. Onderwijscoördinatoren Guy en Diederik, departementshoofd Ann en directeur Lode, dank voor jullie vertrouwen en geloof. Algaeneer Veerle, ik wil je enorm bedanken voor al je praktische hulp en het plezierig samenwerken. Reactoren in elkaar knutselen en opvolgen (al te vaak in het weekend), enorm veel labowerk, events mee organiseren, de pilootverhuis, SAPpen en nog zo veel meer. Jouw 'm van melkkoe' blijft een schitterend relaxatiemedicijn! Caroline, dank voor alle hulp bij het voorbereiden van practica en didactische tips. An, dank voor alle labohulp. Lisbet, muchas gracias por tu apoyo. Bruno, dank voor alle 'rookgasinput'. Heinz & Henk, dank voor de sturing van de laboreactoren. Gerdie, Levi, Xavier, Paul en José, dank voor alle technische hulp in ruil voor algentaartjes. Benny, ons bezoek aan Minister Lieten voor de videopname zal lang bijblijven. Graag bedank ik alle dames van het onthaal, administratieve en onderhoudsmedewerkers.

Thesisstudenten Sem, Anouck, Lies, Wim, Angelique, Cédric, Kim wil ik bedanken voor jullie ijver, inzet en boeiende vragen. I also want to thank the enthousiastic internship students from France and Portugal for contributing to my Ph.D. work: André, Erwan, Gaëlle, Elodie and Marine. Ook aan vele andere ingenieurstudenten, dank voor de boeiende leeromgevingen in het labo en leslokalen.

LabMET colleagues, thank you for the inspiring discussions and feedback, especially all algae researchers Liesje, David, Carlos, Joeri and Alberto, prof. Verstraete and LabMET Ph.D. committee members. Kris en Regine, van harte dank voor alle hulp met de administratieve kantjes. Greet, dank voor de IC-analyses. Tim, dank voor de grafische ontwerpen. Annick, bedankt om je algenenthousiasme te delen tijdens je Diacel-avontuur. Helen en prof. Swennen, dank voor de calciet analyses en hulp bij SEM microscopie. Kristof en Maria, dank voor jullie feedback omtrent statistiek. Jeroen, dank voor het determineren van de algenspecies. Nancy en Peter, dank voor het verkennend testje met MaB-vlokken, visjes en garnalen.

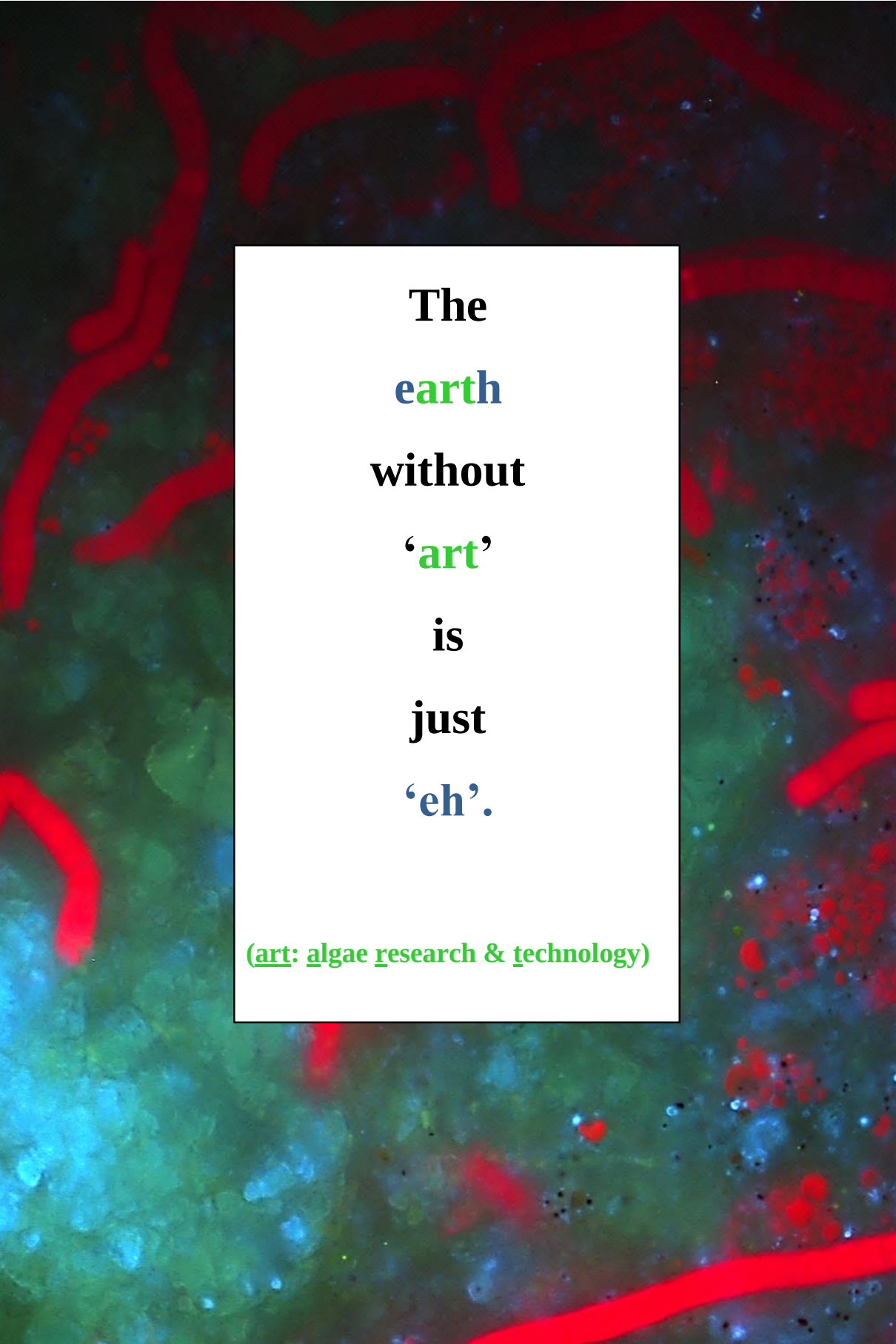
A special thanks to the EnAlgae project leader Shaun from Swansea University and all EnAlgae partners, especially the pilot operators and UGent EnAlgae crew Elien, Kevin, Sue Ellen and Sophie. Tambien quiero agredescer los

investigadores del ESPOL CENAIM y muchos cameroneros para darme la opportunidad de hacer una practica con ustedes.

Zonder (industriële) bedrijven, geen toegepast (industrieel) onderzoek. Een speciaal woord van dank aan de pilootbouwers Lode, Val en Karel Bourez en familie van Bebouwen en Bewaren, en Niels, Nikolas, Ruben en Henk van CATAEL. Stefan, Geert, Laurens en Bart van Inagro, dank voor de infrastructuur en alle praktische hulp! Eric van Crevetec en Leon & Mimi van Aquaculture Farming Technology, dank voor het delen van jullie garnalkweek expertise en infrastructuur. Medewerkers van Aquafin, Stora Enso, VPK, Innova Manure, Alpro en BASF bedank ik graag voor alle afvalwaters en interesse in dit onderzoek. Ook bedank ik Havatex en Sioen voor het toeleveren van staaltjes van industriële stoffen. Dirk en Kaat, jullie waardevolle input en heel enthousiaste hulp met de electrofereses apprecieer ik sterk.

Zonder mentale steun en op tijd een ontspanning, geen doctoraat. Pa, dank om in me te geloven en om altijd voor me klaar te staan met raad en vooral heel veel daad. USB sticks nodig? Voilà, klaar. Terras lekt? Voilà, gefixt. Wat een realisatiekracht! Dikke merci! Marie-Lou, van harte dank voor het luisterende oor, de gouden raad en de lekkere soepjes en etentjes. Ma, vanop een wolkje ben je er altijd bij om te helpen met heel veel geloof, hoop en liefde. De herinneringen hoe je vroeger in de kleuterklas je job met enorm veel plezier deed, blijven inspireren. Valle & Yorick, Luca, Mare & Ilias, dank voor de ontspannende kampeer- en klimtrips in Fontainebleau, de strandwandelingen, de telefoontjes, en zo veel meer. Hilde & Jan, dank voor de koffiekletsjes, de ontspannende tuinbezoekjes en de hulp met het knutselwerk. Isolde & Hermien, de leuke filmavonden moeten we terug meer doen. Inge & Philippe, Hilde & Bart, Kathelijne & Siegfried, dank voor jullie steun. Meter Rita, dank voor het maken van alle MaB-vlokken zeefzakken, voor de traiteurdienst, de wekdienst, je bezoek aan de piloot, de bezorgdheid en vele kaarsjes. Raf en Jeanneke, van harte dank voor alle steun en de gouden tips. Annik & Steph, dank voor de babbels, de gezellige avonden en deugddoende natuurwandelingen. King Arthur, vanaf nu zal je meter je vaker komen bezoeken. Ik kijk er naar uit!

Sofie, februari 2014


The
earth
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(art: algae research & technology)

Picture on previous page:

Image by fluorescence microscopy of MaB-flocs from a SBR treating paper mill wastewater. Red indicates autofluorescence by photosynthetic pigments; blue and green indicate minerals.

CURRICULUM VITAE

Personal information

Name	Van Den Hende, Sofie N.R.
Date of birth	1976/12/28
Address	Amerikalaan 90, B-8530 Harelbeke, Belgium
E-mail	sofie.vandenhende@ugent.be, sofie_vdhende@yahoo.com
Phone	++32 (0)485 153872

Education

2008 - 2014	**Ph.D. candidate in Applied Biological Sciences** Faculty of Bioscience Engineering, Ghent University, Belgium Ph.D. thesis: Microalgal bacterial flocs for wastewater treatment: from concept to pilot scale Promotors: prof. dr. ir. Nico Boon, dr. ir. Han Vervaeren
2008 - 2013	**Doctoral School Training Program** Doctoral School of (Bioscience) Engineering, Ghent University, Belgium
1994 - 1999	**Bio-engineer in agriculture option plant production (distinction)** Faculty of Bio-science Engineering, Ghent University, Belgium Master thesis: Cryopreservation of Thai banana varieties Lab and field work abroad: Maejo University, Thailand Promotors: Prof. dr. P. Vandenberghe, Prof. dr. S. Swennen, dr. B. Panis
1988 - 1994	**High school option Science and Mathematics** Sint-Jan Berchmans Instituut, Avelgem, Belgium

Professional training

Doctoral School Training		**ects**
2012	Bio-imaging, UGent, Belgium	n.a.
2011	Effective Scientific communication, UGent, Belgium	4
2011	Study visit to projects on microalgal treatment of wastewater, Howest, The Netherlands	1
2011	The secret skills of the innovative entrepreneur, UGent, Belgium	3
2011	Didactic competences I, CVO-VIVO, Belgium	5
2011	Psycho-pedagogic competences I, CVO-VIVO, Belgium	6
2011	Effective Scientific Communication: coach, UGent, Belgium	1
2011	Project Management, UGent, Belgium	2
2011	Workshop Algae Harvesting and Processing for Value Added Applications, 2011/05/26-27, ATP, Belgium	2

2011	Teaching skills for assistants, UGent, Belgium	1
2010	Advanced Academic English: Conference Skills - English Proficiency for Presentations, UGent, Belgium	2
2009	Natural Systems for (Waste)Water Treatment, UGent, Belgium	3
2008 - 2012	Educational support – Practica/Theory/Tutorship	32.7
2008	Algae Culture, UGent, Belgium	3

Major other courses

2012	Workshop algae culture, Plymouth Marine Lab, UK
2010	Basis Course Gas Chromatography, Interscience, Belgium
2008 - present	Teacher, CVO-VIVO, Kortrijk, Belgium
2004	Arcview, Syntrawest, Belgium
2004	Seed production, IAP, Argentina
2001	Permaculture, Permaculture Research Institute, Australia
2000	Development aid, Ministry External Affairs, Belgium

Professional activities

2012 - present **Scientific collaborator EnAlgae project / PhD candidate**
Funding: INTERREG IVB NWE, Flemish Government, Province West-Flanders, Howest/UGent
Employer: Enbichem, Howest, Kortrijk, Belgium
LIWET, UGent, Kortrijk, Belgium
Project tasks: - Innovate: design and operation MaB-floc pilot for wastewater treatment, biogas and feed
- Communicate: Pilot Call, events, papers
- Collaborate: overview algae projects, SOP, data exchange, best practice
Student tutor: 1 master thesis, 1 bachelor thesis, 1 integrated project, 4 international internships

2008 - 2012 **Research assistant / PhD candidate**
Funding: Dehousse scholarship of Howest/Ghent Univ.
Employer: LabMET, Ghent University, Gent, Belgium
Enbichem, Howest, Kortrijk, Belgium
PhD research:Microalgal bacterial flocs on lab-scale
Student tutor at Howest: 4 master theses, 4 bachelor theses, 1 internship, 2 research skill reports
Teaching at Howest:
Chemical principles: lab
Applied environmental sciences: lab
Advanced wastewater treatment: lab
Integrated environmental projects: lab, theory
Integrated water policy: theory
Ecology and nature management: theory
Hydrogeology: theory

	Contribution in project proposal writing Reviewer: New Biotechnol., Biotechnol. Bioeng., Ecol. Eng., J. Hazard. Mat., etc.
2007	**Project collaborator - volunteer program, permaculture** Fundacion Verdemillenio, Ecuador
2006	**Collaborator prevention and risk assessment on farms** Project Preventagri, Université Liège, Belgium
2003 - 2004	**Collaborator demonstration of soil erosion measures** Project MESAM, Inagro - Proclam, Belgium
2003 - 2004	**Soil erosion coordinator Province West-Flanders** Inagro - Proclam, Belgium
2002	**Project collaborator/ volunteer agroforestry and agriculture** Fundacion Golondrinas, Fundacion Verdemillenio, Ecuador
2002	**Teacher Mathematics, English** Armada del Ecuador, Liceo Naval, Galapagos, Ecuador
2001	**Collaborator demonstration of soil erosion measures** ALT project, Inagro - Proclam, Belgium
2000	**Project engineer organic fruit culture** Engineers without borders; Fundacion Golondrinas, Ecuador
2000	**Teacher Mathematics, Physics** High School VTI, Belgium

A1 publications

Van Den Hende, S., Beelen, V., Bore, G., Boon, N., Vervaeren, H.. Up-scaling of aquaculture wastewater treatment by microalgal bacterial flocs: from lab reactors to an outdoor raceway pond. Submitted.

Van Den Hende, S., Carré, E., Cocaud, E., Beelen, V., Boon, N.*, Vervaeren, H.*. Treatment of various industrial wastewaters by microalgal bacterial flocs in sequencing batch reactors. Submitted. (*Shared last author)

Van Den Hende, S., Rodrigues, A., Hamaekers, H., Boon, N.*, Vervaeren, H.*. Extracellular carbonic anhydrase enhances calcium removal from paper mill UASB effluent by microalgal bacterial flocs. Submitted. (*Shared last author)

Van Den Hende, S., Claessens, L., De Muylder, E., Boon, N.*, Vervaeren, H.*. Microalgal bacterial flocs originating from aquaculture wastewater treatment as diet ingredient for *Litopenaeus vannamei*. Submitted. (*Shared last author)

Van Den Hende, S., Vervaeren, H., Boon, N., 2012. Flue gas compounds and microalgae: (Bio-)chemical interactions leading to biotechnological opportunities. Biotechnol. Adv. 30, 1405-1425 (IF = 9.4).

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Panis, B., Strosse, H., **Van Den Hende, S.,** Swennen, R., 2002. Sucrose preculture to simplify cryopreservation of banana meristems. Cryoletters 23, 375-384 (IF = 1.1).

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Van Den Hende, S., 2013. Op bezoek bij Nederlandse algentelers. Management & Techniek 2, 39-40.

Van Den Hende, S., 2012. Afvalwater zuiveren met microalgen. Boer en Tuinder 13 juli, 19.

Van Den Hende, S., Vervaeren, H., Boon, N., 2010. Industrial symbiosis: C, N, and P scavenging from sewage and flue gas with algal bacterial flocs. J. Biotechnol. 150, 278.

Van Den Hende, S., 2006. Vallen kan tegenvallen. Agriconstruct 9, 19-23.

Oral presentations

Van Den Hende, S., Beelen, V., Carré, E., Bore, G., Vervaeren, H., 2013. Treatment of aquaculture wastewater in a MaB-floc pilot reactor. Official EnAlgae Pilot launch, 2013/3/26, Beitem, Belgium. Oral presentation.

Van Den Hende, S., Beelen, V., Carré, E., Vervaeren, H., 2012. MaB-vlokken voor afvalwaterzuivering: van labo tot pilootschaal. Aquacultuur Technische Adviesraad, 2012/10/26, Inagro, Beitem, Belgium. Oral presentation (invited speaker).

Van Den Hende, S., Beelen, V., Carré, E., Vervaeren, H., 2012. Voorbereiding MaB-vlokken pilootreactor: labo-experimenten, bezoeken en ontwerp. EnAlgae info-session, 2012/10/10, Howest, Kortrijk, Belgium. Oral presentation.

Van Den Hende, S., Beelen, V., Carré, E., Vervaeren, H., 2012. EnAlgae pilot reactors for wastewater treatment and algal bioenergy production. Wastewater course for students industrial Engineering Environmental Sciences, 2012/09/13, EME, Rennes, France. Oral presentation (invited speaker).

Van Den Hende, S., Beelen, V., Vervaeren, H., Boon, N., 2012. Wastewater treatment with MaB-flocs: from lab to pilot scale. Mini-symposium Howest & University Zjut China, 2012/06/27, Howest, Kortrijk, Belgium. Oral presentation (invited speaker).

Van Den Hende, S., Vervaeren, H., Rodrigues, A., Hamaekers, H., Boon, N., 2012. Calcium removal from paper mill UASB effluent using microalgal bacterial flocs. Young Algaeneers Symposium, Wageningen, The Netherlands, 2012/6/14-16. Book of Abstracts p26. Oral presentation.

Vervaeren, H., **Van Den Hende, S.**, 2012. RRB-8: Wastewater treatment with MaB-flocs: from lab to pilot scale. International conference on Renewable Resources and Biorefeneries, 2012/6/4-7, Toulouse, France. Book of Abstracts p84. Oral presentation.

Van Den Hende, S., 2012. EnAlgae: Energetic Algae. Field visit to algae pilots, 2012/5/24-25, University Wageningen, Wageningen, The Netherlands. Oral presentation.

Van Den Hende, S., Vervaeren, H., Boon, N., 2012. Flue gas compounds and microalgae: (Bio-)chemical interactions leading to biotechnological opportunities. 2nd Annual World Congress of Bioenergy, 2012/4/25-28, Xi'An, China. Book of Abstracts p215. Oral presentation (invited speaker) and co-chair Young Scientist Session.

Van Den Hende, S., Vervaeren, H., Boon, N., 2012. Afvalwaterzuivering met MaB-vlokken: van labo tot pilootschaal. VCM Workshop Manure Treatment, 2012/3/20, Antwerpen, Belgium. Oral presentation (invited speaker).

Van Den Hende, S., Vervaeren, H., Desmet, S., Boon, N., 2011. Bioflocculation of microalgae and bacteria combined with flue gas to improve sewage treatment. Cornet Albaqua Final Conference, 2011/12/7, Munich, Germany. Oral presentation (invited speaker).

Van Den Hende, S., Vervaeren, H., Boon, N., 2011. Albapro Algae-bacteria operation processes for wastewater treatment and biomass production: Cornet project proposal. Workshop VAP, 2011/9/27, Antwerpen, Belgium. Oral presentation (invited speaker).

Van Den Hende, S., Vervaeren, H., Desmet, S., Boon, N., 2011. Bioflocculation of microalgae and bacteria combined with flue gas to improve sewage treatment. 2nd IWA BeNeLux Regional Young Water Professionals Conference, 2011/9/22, Leuven, Belgium. Abstracts. Oral flash presentation.

Van Den Hende, S., Desmet, S., Vervaeren, H., Boon, N., 2011. Bioflocculatie en rookgasinjectie ter optimalisatie van waterzuivering met microalgen. Workshop VAP Oogsten van algen, 2011/3/31, Gent, Belgium. Oral presentation (invited speaker).

Van Den Hende, S., Vervaeren, H., Desmet, S., Boon, N., 2011. Optimalisatie van de behandeling van rioolwater met MaB-vlokken en rookgas. Howest/K.U.Leuven studiedag 'Algen voor waterzuivering, doenbaar?', 2011/3/25, Kortrijk, Belgium. Oral presentation (invited speaker).

Van Den Hende, S., Vervaeren, H., Desmet, S., Boon, N., 2010. Carbon and nutrient scavenging from sewage and flue gas with MaB-flocs. AquaFUELs Roundtable meeting, 2010/10/21-22, Brussels, Belgium. Proceedings p44. Oral presentation.

Boon, N., **Van Den Hende, S.**, 2010. Microalgen bacterïen interacties voor de duurzame verwijdering van gassen. KVIV studiedag 'Potentieel van algen voor de industrie', 2010/11/30, Berchem, Belgium. Samenvattingen. Oral duo-presentation (invited speaker).

Poster presentations

Van Den Hende, S., Beelen, V., Vervaeren, Bore, G., Cocaud, E., Begué, M., Boon, N., 2013. MaB-flocs for wastewater treatment: from lab to pilot scale. AOG Renew Meeting Resource and energy recovery from water and biomass, 2013/6/13, Kortrijk, Belgium. Poster.

Van Den Hende, S., Beelen, V., Vervaeren, H., 2012. MaB-flocs for wastewater treatment: from lab to pilot scale. Dag van de Biotechnology, 2012/6/23, Gent, Belgium. Poster.

Van Den Hende, S., Vervaeren, H., Boon, N., 2012. Afvalwaterzuivering met MaB-vlokken: van labo tot pilootschaal. VCM Workshop Manure Treatment, 2012/3/20, Antwerpen, Belgium. Poster.

Van Den Hende, S., Beelen, V., Vervaeren, H., 2012. MaB-flocs for wastewater treatment: from lab to pilot scale. Cleantech Day, 2013/6/15, Poperinge, Belgium. Poster.

Van Den Hende, S., Beelen, V., Vervaeren, H., 2012. MaB-flocs for wastewater treatment: from lab to pilot scale. Themadag 'Landbouw als producent van groene grondstoffen', 2012/12/7, Terneuzen, The Netherlands. Poster.

Van Den Hende, S., Vervaeren, H., Boon, N., 2012. Afvalwaterzuivering met MaB-vlokken: van labo tot pilootschaal. VCM Workshop, 2012/03/20, Antwerpen, Belgium. Poster.

Van Den Hende, S., Vervaeren, H., Desmet, S., Boon, N., 2010. Industrial symbiosis: C, N and P scavenging from sewage and flue gas with algal bacterial flocs. IBS2010, 2010/9/14-18. Rimini, Italy. Special Proceedings in J. Biotechnol. 150, 278. Poster.

Van Den Hende, S., Vervaeren, H., Desmet, S., Boon, N., 2011. Flue gas to improve sewage treatment with MaB-flocs: optimisation of the gas flow rate. 1st International Symposium on Microbial Resource Management, 2011/6/30 – 7/1, Gent, Belgium. Abstracts p52. Poster.

Vervaeren, H., **Van Den Hende, S.,** Daels, T., 2010. Environmental Sciences Research. Cleantech Summer Event, 2010/07/01, Oostende, Belgium. Abstracts. Poster.

Van Den Hende, S., Vervaeren, H., Maes, G., Boon, N., 2010. MaB-flocs for a more sustainable wastewater treatment. Neptune/Innowatech End-user Conference, 2010/1/27 Gent, Belgium. Poster.

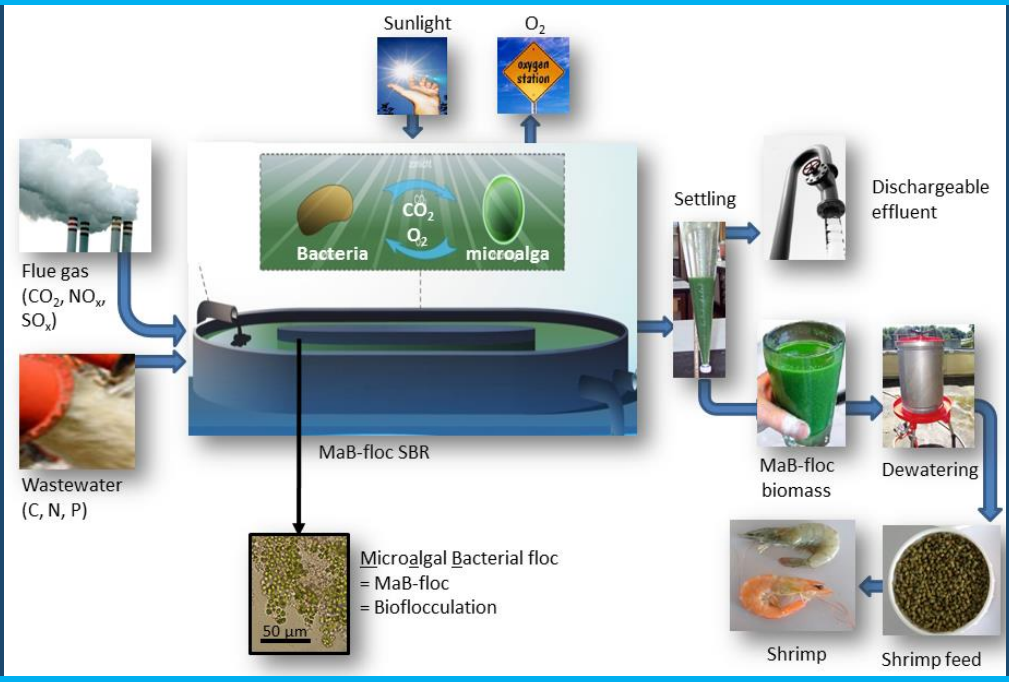
Van Den Hende, S., Arends, J., Schouppe, J., De Schamphelaire, L., Verstraete, W., Boon, N., Vervaeren, H., 2009. Algen voor zuivering en energieproductie. 4e Dag van het Onderzoek, 2009/11/17, Gent, Belgium. Poster.

Organization of events

UGent: Mini-symposium: Algen voor waterzuivering, 2014/02/27, Wevelgem, Belgium
Howest: EnAlgae Official Pilot Launch, 2013/03/26, Roeselare, Belgium
EnAlgae Info-sessie, 2012/10/10, Kortrijk, Belgium
Field visit to algae pilots, 2012/5/24-25, Hallum/Wageningen/Lelystadt, The Netherlands
Inagro: Several events including open days, field visits, info-sessions

Scientific awards

Award for Ph.D. research on 'Water and its availability worldwide - 2012', from the Ernest du Bois Fund, managed by the King Baudouin Foundation, Belgium.



Although sunlight-powered microalgal technology is promising for a cradle-to-cradle approach of wastewater treatment based on photosynthetic aeration, its implementation is hampered by high costs for microalgae harvesting. To address this challenge, this work proposes a novel concept based on bioflocculation: microalgal bacterial flocs in sequencing batch reactors (MaB-floc SBRs). Gravity settling of MaB-flocs enables a costless separation of biomass from the treated wastewater. The concept shows successful results in lab-scale SBRs treating various (agro-)industrial wastewaters and sewage. Up-scaling to an outdoor MaB-floc raceway pond treating aquaculture wastewater, a worldwide exclusivity, is promising especially regarding MaB-floc settling and harvesting. Flue gas injection is needed to enhance the treatment of aquaculture wastewater outdoors. This flue gas contains several compounds and presents various biotechnological opportunities. The dried MaB-floc biomass can be used as shrimp feed ingredient enhancing shrimp pigmentation. Further optimisation is needed to set the stage for the industrial implementation of MaB-floc SBRs for wastewater treatment.

ISBN 978-9-0598968-4-0
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UNIVERSITY press