Mälardalen University Press Dissertations
No. 196

MICROBIOLOGICAL ANALYSIS OF MUNICIPAL WASTEWATER TREATING PHOTOBIOREACTORS

Ivo Krustok

2016

MÄLARDALEN UNIVERSITY
SWEDEN

School of Business, Society and Engineering

ISBN 978-91-7485-251-6
ISSN 1651-4238
Printed by Arkitektkopia, Västerås, Sweden

Mälardalen University Press Dissertations
No. 196

MICROBIOLOGICAL ANALYSIS OF MUNICIPAL WASTEWATER TREATING PHOTOBIOREACTORS

Ivo Krustok

Akademisk avhandling

som för avläggande av teknologie doktorsexamen i energi- och miljöteknik vid Akademin för ekonomi, samhälle och teknik kommer att offentligen försvaras fredagen den 29 januari 2016, 09.15 i Paros, Mälardalens högskola, Västerås.

Fakultetsopponent: Associate Professor Raul Muñoz, University of Valladolid

Akademin för ekonomi, samhälle och teknik

Abstract

Microalgae reactors, commonly known as photobioreactors, have become increasingly popular as an alternative for wastewater treatment. These systems reduce pollutants and remove nutrients such as nitrogen and phosphorous compounds from wastewater utilizing microalgae and bacteria. The biomass produced in the reactors can potentially be used to produce biofuels and decrease some of the energy demands of the process.

Wastewater treating photobioreactors are a relatively new technology and many aspects of their microbiology need further study. This thesis presents a broad overview of the algal and bacterial communities present in these systems by looking at the most important species, metabolic pathways and growth dynamics of both algae and bacteria.

The experiments presented in this thesis were conducted using municipal wastewater from the Västerås wastewater treatment plant. The wastewater was inoculated with algae from Lake Mälaren and compared to non-inoculated reactors. Overall, the inoculated reactors demonstrated better algal growth than those that were not inoculated. The tested systems also removed much of the ammonium and phosphorous present in the wastewater.

The dominant algae in the tested systems belonged to the genera *Scenedesmus, Desmodesmus* and *Chlorella*. In addition to algae, the systems contained a large number of bacteria, mostly from the phyla *Proteobacteria* and *Bacteroidetes*.

The algal photobioreactors contained a lower abundance of genes related to nitrogen metabolism, virulence and antibiotic resistance compared to the initial wastewater, showing that a shift in the bacterial community had occurred. The bacteria found in the systems were shown to be involved in synthesis of vitamins essential for algae growth such as vitamin B12, suggesting cooperation between the bacteria and algae.

ISBN 978-91-7485-251-6
ISSN 1651-4238

Summary

With the growing human population, the increasing amount of wastewater produced daily presents a challenge to the environment. When designing systems and infrastructure for wastewater treatment, we are limited by the ever-growing demands to reduce energy use. Currently used systems have many shortcomings when faced with modern treatment criteria and energy use restrictions. Microalgae reactors, commonly known as photobioreactors, have been suggested as an alternative. These systems use microalgae and bacteria to reduce pollutants and remove nutrients such as nitrogen and phosphorous compounds.

Water treatment systems using photobioreactors are a relatively new technology and several aspects of their biology have yet to be studied in detail. This thesis presents a broad overview of the algal and bacterial communities present in these systems. In addition to looking at the most important species, metabolic pathways and growth dynamics of both algae and bacteria, this thesis also analyses water purification dynamics.

Municipal wastewater from the Västerås wastewater treatment plant was inoculated with algae from Lake Mälaren and several different experiments were conducted with reactor volumes from 250 ml to 20 L. The inoculated reactors demonstrated better algal growth than those that were not inoculated. All tested systems were also successful in removing ammonium and phosphorous from the wastewater.

The dominant algae growing in the studied photobioreactors belonged to the genera *Scenedesmus, Desmodesmus* and *Chlorella.* In addition to algae, the systems contained a large number of bacteria, mostly belonging to the *Proteobacteria* and *Bacteroidetes* phyla. These were shown to be involved in synthesis of vitamins essential for algae growth such as vitamin B12, which is not commonly synthesized by algae, suggesting cooperation between the bacteria and algae.

In addition to vitamin synthesis, algal photobioreactors contained a lower abundance of genes related to nitrogen metabolism, virulence and antibiotic resistance compared to the initial wastewater, showing that a shift in the bacterial community had occurred.

Overall, the information regarding algal and bacterial populations and metabolic genes presented in this thesis is important for the development of tools for the control and monitoring of full-scale wastewater treating photobioreactor plants.

Sammanfattning

I takt med att världens befolkning ökar, så produceras dagligen allt mer avfall. Detta kan orsaka stora problem för miljön. När det byggs nya system för vattenrening behöver vi även ta hänsyn till kravet att minska energiåtgången. Dagens vattenreningssystem har vissa tillkortakommanden när det gäller reningsnivåer och energianvändning. Ett alternativ till dagens system, kan vara fotobioreaktorer, dvs. vattenrening med hjälp av mikroalger. Dessa system använder mikroalger och bakterier för att rena vattnet från föroreningar, kväve och fosfor.

Vattenrening med fotobioreaktorer är en relativt ny teknik. Flera aspekter gällande biologin i dessa system har ännu inte studerats i detalj. Den här avhandlingen presenterar en översikt av de alger och bakterier som är aktiva i fotobioreaktorer. Andra viktiga aspekter som tillväxt, arter samt vattenreningsförmåga har också studerats.

Ett antal försök genomfördes där alger från Mälaren tillsattes i vatten från Västerås kommunala vattenreningsanläggning. Storleken på försöken varierade mellan 250 ml och 20 liter. Det visade sig att algerna hade en bra tillväxt samt att mängden ammonium och fosfor minskade i vattnet under försöksperioden.

De alger som tillväxte mest i studien tillhörde *Scenedesmus, Desmodesmus* och *Chlorella*. Förutom alger tillväxte även ett stort antal bakterier från grupperna *Proteobacteria* and *Bacteroidetes*. Dessa bakterier visade sig syntetisera viktiga vitaminer, t.ex. vitamin B12, som algerna normalt inte kan syntetisera själva.

Sammanfattningsvis, så presenterar denna avhandling viktig information gällande alger och bakterier i en fotobioreaktor. Informationen kan vara ett viktigt bidrag till framtida utveckling av storskaliga fotobioreaktorer för vattenrening.

List of papers

I. Krustok I., Odlare M., Shabiimam M.A., Truu J., Truu M., Ligi T., Nehrenheim E., 2015. Characterization of algal and microbial community growth in a wastewater treating batch photo-bioreactor inoculated with lake water. Algal Research, Volume 11, pp 421-427.

II. Krustok I., Odlare M., Truu J., Nehrenheim E., 2015. Inhibition of nitrification in municipal wastewater treating photobioreactors: effect on algal growth and nutrient uptake. Accepted for publication in Bioresource Technology.

III. Krustok I., Odlare M., Truu M., Truu J., Ligi T., Tiirik K., Nehrenheim E., 2015. Effect of lake water on algal biomass and microbial community structure in municipal wastewater based lab-scale photobioreactors. Applied Microbiology and Biotechnology, Volume 99, Issue 15, pp 6537-6549.

IV. Krustok I., Oopkaup K., Truu J., Odlare M., Nehrenheim E., 2015. Comparative analysis of the metagenomes extracted from wastewater treating photobioreactors. Manuscript draft.

Author's Contribution

I. Participated in the planning and conducted all of the experiments. Performed most of the laboratory analyses, data evaluation and writing.

II. Performed a majority of the planning, the experiments, the laboratory analyses, data evaluation and writing.

III. Participated in the planning and conducted all of the experiments. Performed a majority of the laboratory analyses, data evaluation and writing.

IV. Participated in the planning and conducted all of the experiments. Performed a majority of the laboratory analyses, and much of the data evaluation and writing.

List of Papers Not Included

I. Krustok I., Diaz J.G., Odlare M., Nehrenheim E., 2015. Algae biomass cultivation in nitrogen rich biogas digestate. Water Science & Technology.

II. Krustok I., Nieto J.G.D., Odlare M., Nehrenheim E., 2014. Algae Biomass Cultivation in Ammonium Rich Reject Water – The Potential for Simultaneous Wastewater Treatment and Energy Recovery. Presented at the 5th International Symposium on Energy from Biomass and Waste, Venice, Italy.

III. Krustok I., Nehrenheim E., Odlare M., Shabiimam M.A., Truu J., Ligi T., Truu M., 2014. Characterization of algal and microbial community dynamics in a wastewater photo-bioreactor using indigenous algae from Lake Mälaren. Presented at the 4th international Conference on Algal Biomass, Biofuels and Bioproducts, Santa Fe, USA.

IV. Nehrenheim, E., Odlare, M. Krustok, I., Olsson J., Ribé V., Shabiimam M.A., Diaz J.G., Nordlander E., 2013. ACWA - algae cultivation for simultaneous water treatment and biogas substrate production. Poster at the 14th International Waste management and Landfill Symposium.

V. Ribé V., Nehrenheim E., Shabiimam M.A., Krustok I., Thorin E., 2013. AlTox: biomass production using potentially toxic landfill leachates as substrates for algae cultivation. Presented at the 14th International Waste management and Landfill Symposium.

VI. Shabiimam M.A., Krustok I., Nehrenheim E., Odlare M., 2013. Microalgae cultivation for potential nutrient and heavy metal reduction in landfill leachate. Presented at the 14th International Waste management and Landfill Symposium.

VII. Krustok I., Truu J., Truu M., Preem J-K., Nehrenheim E., Odlare M., Mander Ü. 2012. Bacterial Community Activity, Structure and Succession in Hybrid Constructed Wetland Treating Domestic Grey Water. Presentation at the 1st Congress of Baltic Microbiologists, Riga, Latvia

Contents

List of Abbreviations

ANOVA – Analysis of Variance
ARG – Antibiotic Resistance Gene
BOD - Biochemical Oxygen Demand
DOC – Dissolved Organic Carbon
FAME - Fatty Acid Methyl Ester
LWR – Lake Water Reactor
M5NR - M5 Non-Redundant Protein Database
NH_4-N – Ammonium Nitrogen
NO_2-N – Nitrite Nitrogen
NO_3-N – Nitrate Nitrogen
OD – Optical Density
PAR - Photosynthetically Active Radiation
PCR-DGGE - Polymerase Chain Reaction Denaturating Gradient Gel Electrophoresis
PE – Purification efficiency
RT-PCR – Real-Time Polymerase Chain Reaction
SSU - SILVA Small Subunit
STAMP - Statistical Analysis of Metagenomic Profiles
SWR – Sterilized Wastewater Reactor
TOC – Total Organic Carbon
TP – Total Phosphorous
TWR – Tap Water Reactor
VFDB - Virulence Factor Database
WWR – Wastewater Reactor
WWTP – Wastewater Treatment Plant

Acknowledgements

The research presented in this thesis was conducted at the School of Business, Society and Engineering, Mälardalen University, Västerås, Sweden with financial support from the Knowledge Foundation (2011006), VINNOVA (2012-01243), SVU (12-123), Purac, Mälarenergi and the Ministry of Education and Research of the Republic of Estonia (grants IUT2-16 and 3.2.0801.11-0026). First things first - I would like to thank my supervisors Monica, Emma and Jaak, who have given me large portions of their valuable time so that this work could be as good as it is. I have learned a lot from all three of you and felt a strong connection between us. I will always value the work we did together.

I also owe much to my co-authors Marika Truu, Shabiimam M.A., Kristjan Oopkaup, Teele Ligi and Kertu Tiirik. You have helped make this thesis as it is today. A special thanks goes to Veronica Ribé, Olga Chusova, Javier Campillo, Jesper Olsson, Sebastian Schwede and Anbarasan Anbalagan from Mälardalen University, Mae Uri from the Institute of Ecology and Earth Sciences at the University of Tartu, Viktor Sjöberg from Örebro University and David Ribé for helping me with my experiments and giving assistance and advice.

I would also love to thank my office mates – both current and previous – Mehmet Bulut, Nima Ghaviha and Lokman Hosain. Many kind words also go to all the other amazing students and employees who made work in MDH better every day. I am not sure I would have been able to finish all my work without your support.

I owe a lot of gratitude to my wife who has supported me during my research and has understood if I cannot be home some evenings or weekends. I am also very grateful to my parents, who have always helped me follow my passions and have understood why I have only been able to visit home during the summer or over Christmas.

1 Introduction

Wastewater treatment is a complicated and costly process. This is in large part due to the enormous amount of wastewater produced globally every day. As modern wastewater treatment plants need to reduce pollutants to an ever lower level and do so at a lower cost, many have started to look for alternatives to the commonly used treatment processes. Photobioreactors are bioreactors specifically designed to grow photosynthetic organisms like algae. While their main use has been biomass production (Benemann, 2013), they are also being investigated for use in wastewater treatment (Fig. 1), due to the ability of microalgae to take up nutrients from water. Compared to photobioreactors growing a single species of algae, algal systems for wastewater treatment contain a consortia of different microbes as wastewater itself contains a large variety of different microorganisms (Ju et al., 2014). In monoculture cultivation this would be considered contamination, however it can be beneficial for wastewater treatment as the microbial processes are more robust in terms of the metabolic pathways in the system (Muñoz and Guieysse, 2006). Muñoz and Guieysse (2006) have also pointed out that mixed consortium photobioreactors have a higher capacity for producing biomass than pure cultures, which could be useful for production of biodiesel (Rawat et al., 2013; Sivakumar et al., 2012) or biogas (Mussgnug et al., 2010; Olguín, 2012; Passos et al., 2013) to offset the energy requirements of the process.
Because biotechnological systems rely on microbial communities to achieve their goals, there is a growing need to understand the interactions between algal and bacterial communities to enhance the available technologies and to create new ones (Kouzuma and Watanabe, 2015). In the past few years several studies have provided valuable insights into the communities in multi-species photobioreactors using molecular methods (Carney et al., 2014; Krohn-Molt et al., 2013). For example Carney et al., 2014 described the microbiome of a municipal wastewater treating photobioreactor prototype, revealing the key species present. Similarly, Krohn-Molt et al., 2013 analysed the metagenome of algae associated biofilms and described some of the bacterial-algal interactions found.

More information is also being generated about the synergistic, parasitic and competitive interactions between bacteria and algae in both natural and biotechnological environments (Amin et al., 2012; Kouzuma and Watanabe, 2015). In wastewater treatment systems bacteria and algae work together to break down organic compounds, take up nutrients and reduce pollutants.

(Muñoz and Guieysse, 2006). One documented form of co-operation is related to gas exchange. The algae produce oxygen, which the bacteria can use to break down organic compounds to CO_2, which the algae can then use to produce further oxygen. Bacteria are also known to provide microalgae with important nutrients such as vitamin B12 (Croft et al., 2005). Inter-kingdom signalling has also been described between algae and bacteria (Amin et al., 2012), suggesting that both kingdoms have ways of understanding which types of bacteria or algae are present locally. There are also reports of horizontal gene transfer between algae and bacteria (Brembu et al., 2014; Moszczyński et al., 2012) suggesting that genetic information is also being exchanged.

Figure 1. Experimental wastewater treating photobioreactor system combining microalgae with the activated sludge process in Västerås wastewater treatment plant, Sweden.

Despite these advances there is still much to be learned about the composition of the microbial communities in these systems. There is also very little known about the available metabolic pathways and functional genes and how they could be used in profitable ways when developing new biotechnological approaches (Kouzuma and Watanabe, 2015). Many authors have also argued that studying the microbiology of photobioreactors could one day help create consortia with desirable treatment capabilities and good biomass growth (González-Fernández et al., 2011; Kouzuma and Watanabe, 2015; Lakaniemi et al., 2012; Subashchandrabose et al., 2011). Subashchandrabose et al. (2011) concluded that understanding the community relationships is crucial when

treating different wastewaters with microalgae so that more biomass can be produced and the pollutants degraded. This information is not only important for the control of these systems but also for the development of specific molecular probes that will allow for rapid monitoring of the communities and metabolic pathways in the reactors (Carney et al., 2014).

1.1 Objectives

The overall objective of this thesis was to study the microbiology and performance of municipal wastewater treating mixed consortia photobioreactors. The thesis provides results from experiments conducted with different concentrations of wastewater, inoculation techniques, and methodologies to study the community dynamics and interactions, pollutant removal and nitrogen transformations.

The specific objectives were to (1) investigate the dynamics of the microbial and algal community in a wastewater photobioreactor after introduction of indigenous algae from a nearby inland freshwater lake sampled during different seasons and how the nitrogen and phosphorous concentrations change throughout the algae cultivation process (Paper I), (2) study nitrogen transformation pathways in wastewater treating photobioreactors (Paper II and Paper IV), and (3) compare the microbial communities in inoculated and non-inoculated photobioreactors to identify connections between the community composition, biomass growth and treatment parameters (Paper III and Paper IV).

1.2 Research questions

The research questions studied in the included papers were as follows:

Q1: How does lake water inoculation affect algal growth in wastewater and does the inoculant sampling season affect growth? (Paper I)

Q2: How does the algal growth affect bacterial populations? (Papers I and III - IV)

Q3: Does the inhibition of nitrification have an effect on the growth of algae and the uptake of phosphorous and nitrogen? (Paper II)

Q4: What effect does lake water inoculation have on algae and bacteria communities and nitrogen metabolism? (Papers III-IV)

Q5: What metabolites are the bacteria producing and are they beneficial for the algae? (Paper III)

Q6: Do algae and bacteria reduce heavy metals in the water phase? (Paper III)

Q7: Is pathogen gene abundance significantly reduced in photobioreactors? (Papers III-IV)

Q8: What happens to antibiotic resistance genes in photobioreactors? (Paper IV)

1.3 Thesis structure

This doctoral thesis is comprised of four scientific papers (Paper I-IV) which share the common goal of describing parameters connected to the performance of wastewater treating photobioreactors, their effects on the emerging microbial population and how the resulting algal-bacterial population affects the system (Fig. 2).

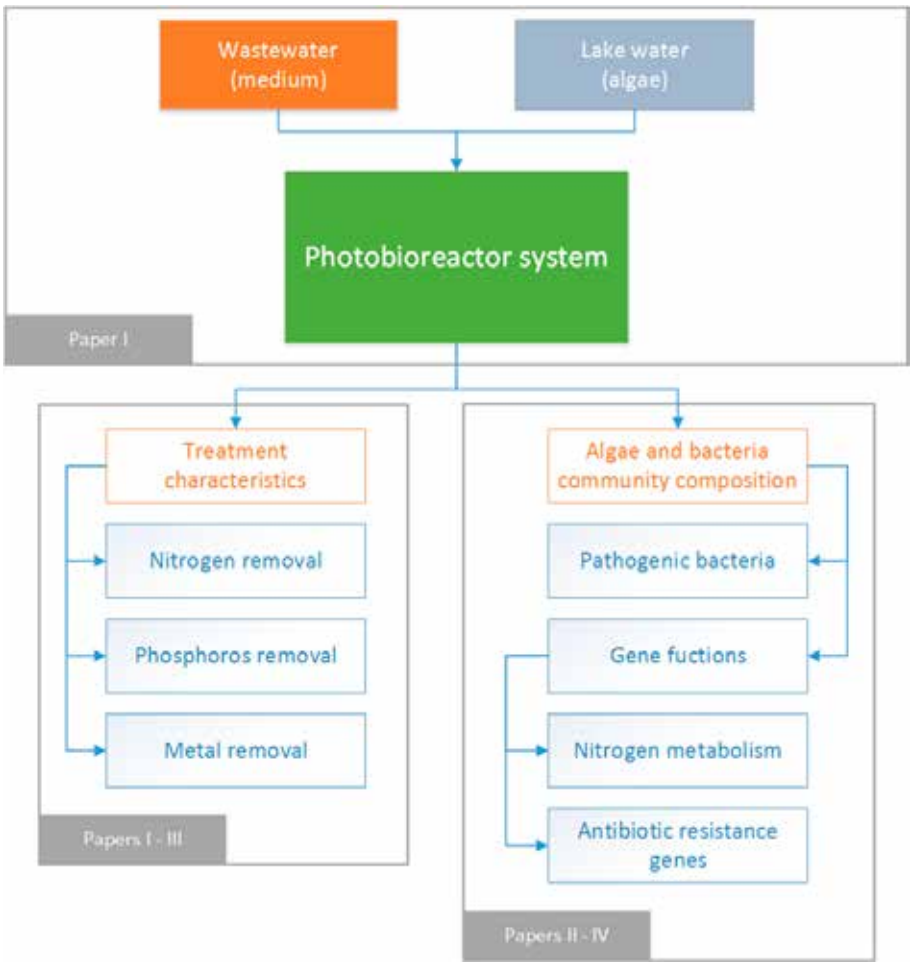

Figure 2. Overview of the different parameters and characteristics of photobioreactors studied in the different papers collected in this thesis.

The doctoral thesis is comprised of the following chapters:

Chapter 1 Introduction to the thesis, presenting its objectives, author contributions and outline.

Chapter 2 Literature review of the topics discussed in the thesis.

Chapter 3 Methodologies used in the studies.

Chapter 4 Results and discussion of the studies.

Chapter 5 Main conclusions of the thesis.

Chapter 6 Limitations and possible directions for future work.

2 Literature review

2.1 Microalgae for wastewater treatment

Microalgae have been used for tertiary wastewater treatment for several decades. However, over the last 10 years research has shown that microalgae technology can also be used for secondary wastewater treatment to replace expensive and old technologies such as the activated sludge process. Because of their ability to reduce biochemical oxygen demand (BOD), remove nutrients, heavy metals and pathogens as well as heterotrophic pollutants, they fulfil the main requirements for hazardous contaminant treatment (Muñoz and Guieysse, 2006).

Due to the availability of wastewater near human habitats it is a very accessible medium for algae growth. Algae primarily need carbon, nitrogen, and phosphorus to grow. Trace amounts of silica, calcium, magnesium, potassium, iron, manganese, sulphur, zinc, copper, and cobalt are also needed (Knud-Hansen, 1998). According to the Redfield ratio, the optimal nitrogen to phosphorous ratio for algae growth is 16:1. Municipal wastewater often has a similar nitrogen to phosphorous ratio, making it a good candidate for a nutrient solution (Christenson and Sims, 2011). Wastewater is also rich in trace elements, giving algae a source of micronutrients.

There is also a large community of microorganisms present in wastewater (Ju et al., 2014), which interacts with the algae, assisting in the water treatment. Photobioreactors take advantage of these algae/bacteria consortia to break down organic compounds, remove nutrients from the water and reduce the amount of pathogens and pollutants (Muñoz and Guieysse, 2006).

While microalgae wastewater treatment systems have shown a lot of promise for removal of nutrients (de-Bashan et al., 2004; Wang and Lan, 2011) and heavy metals (Romera et al., 2007), there is limited information about how they deal with persistent pollutants such as pharmaceutical and hormone residues. In a recent study, Escapa et al., (2015) demonstrated around 70% removal of paracetamol and salicylic acid in a flask experiment using *Chlorella sorokiniana*. They also noted that removal rates for salicylic acid were 2.3 times higher than for paracetamol.

Algal systems with bacteria are also known to produce a larger amount of algal biomass compared to pure cultures and bacteria based activated sludge systems. This is understandable as in natural environments microalgae and

cyanobacteria live and interact with other microbes (Amin et al., 2012; Subashchandrabose et al., 2011). This presents a possibility of recovering some of the energy that is needed to run the process as the biomass could be used to produce energy (Christenson and Sims, 2011). Additionally, different metabolites could be produced to offset the cost of the process (Subashchandrabose et al., 2011).

2.1.1 Examples of wastewater treating photobioreactors

There are many parameters to consider when constructing photobioreactors for wastewater treatment, regardless of the scale. Most commercial photobioreactors are constructed to deal with nutrient solutions and monocultures and may not be suitable for use with wastewater without prior modification. There are however a wide range of photobioreactor configurations available to choose from (Behrens, 2011). Unfortunately many of the systems studied are lab-scale and for optimised conditions, with only few demonstrating the feasibility of wastewater based algae systems in outdoor environments (Zhou et al., 2014)

At the simple end of the spectrum there are single-step systems that are inoculated with a single algae species, such as the one proposed by Henkanatte-Gedera et al., (2015). In a lab-scale wastewater treatment system inoculated with *Galdieria sulphuraria*, they demonstrated removal rates for BOD, N and P of 14.93, 7.23 and 1.38 mg L^{-1} d^{-1} respectively.

Carney et al., (2014) describe OMEGA, a prototype system built at the Southeast Wastewater Treatment Plant in San Francisco, CA. The system had a volume of 1600L and contained four floating photobioreactors through which the water was circulated at 10 cm s^{-1}. Around 5% of the algae culture was continuously diverted so that algae could be harvested, oxygen could be removed and CO_2 added. While 1600 L is still not large enough for a big community, this study is much more applicable for future large scale use than data collected from lab scale studies.

Over time, more complex systems have been proposed. Alcántara et al., (2015) proposed an algae-bacterial photobioreactor with anoxic and aerobic tanks with separate hydraulic and sludge retention times. The system supported the removal of 86-90% of total organic carbon (TOC), 57-98% of inorganic carbon and 68-79% of total nitrogen. The recycling of biomass inside the system resulted in low effluent total suspended solids concentrations.

2.2 Microbial communities in wastewater treating photobioreactors

The composition of the algae and bacteria community has a large effect on both the treatment capabilities of a photobioreactor and the biomass production (Kesaano and Sims, 2014). This has not gone unnoticed in the scientific community and while much of the research on mixed culture photobioreactors was done before widespread use of molecular biology methodologies (Ferrero et al., 2012), there has been increasing interest in studying these systems with a focus on the microbiology (Kouzuma and Watanabe, 2015).

With recent developments in novel molecular biology methods such as metagenome sequencing, there is a growing community of researchers studying the microbial communities and the interactions between micro-organisms in mixed culture photobioreactors (Carney et al., 2014; Kouzuma and Watanabe, 2015; Krohn-Molt et al., 2013; Lakaniemi et al., 2012). The information revealed in these studies could enable increased biomass production and water treatment efficiency and fine-tuning to specific use cases (Lakaniemi et al., 2012; Subashchandrabose et al., 2011). There is also interest in developing molecular probes that can simplify molecular analysis, allowing for rapid monitoring of the communities present (Carney et al., 2014). Since the cost and complexity of these analyses is decreasing, plant operators can benefit from this information and diagnose faults in their biological processes more quickly.

2.2.1 Algal communities

In natural environments such as lakes and seas, algae usually exist in large communities with many different species present. Depending on the environmental conditions these communities change over seasons and over years (Willén, 1987). This is however generally not the case in photobioreactors, where many systems have been built to grow monocultures with *Chlorella* and *Scenedesmus* being the most commonly used genera – this is done so that specific strains selected for their characteristics and ideal environmental parameters can be chosen, maintained and controlled (Wu et al., 2014). In wastewater treatment these strains (Fig. 3) are often selected for their ability to grow in specific wastes or reduce the levels of the toxic compounds present (Muñoz and Guieysse, 2006).

Many waste streams contain compounds that are toxic to microalgae such as heavy metals, herbicides (Suresh Kumar et al., 2014) and organic pollutants (Chen and Lin, 2006), so selecting suitable strains for a particular waste stream is important (Muñoz and Guieysse, 2006).

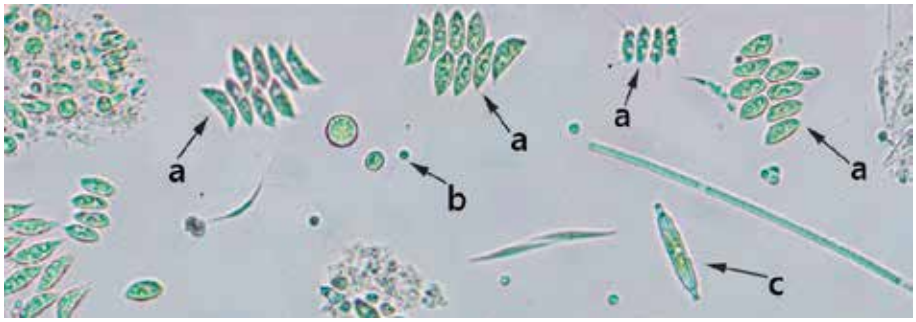

Figure 3. Compilation image of the most common microalgae seen in wastewater photobioreactors inoculated with Lake Mälaren water in experiments conducted in Paper I. Various *Scenedesmus sp.* are marked with a, *Chlorella sp.* is marked with b and a diatom species is marked with c.

Wu et al., (2014) compiled a review of microalgal species used in wastewater treatment and biomass production and concluded that photoautotrophic unicellular green microalgae are tolerant to many wastewater conditions and are therefore the most commonly used. In addition to photoautotrophic microalgae, mixotrophic microalgae are also used. Depending on the organic matter in different types of wastewater, Wu et al., (2014) also noted that some microalgae species such as *Botryococcus braunii, C. vuglaris* and *S. obliquus* may grow photoautotrophically in one specific wastewater and mixotrophically in another. This is also reflected in the literature as many studies have used these strains to inoculate their photobioreactors (Cabanelas et al., 2013; Cho et al., 2013; Escapa et al., 2015; González et al., 2008; Park et al., 2012).

In addition to using monocultures in wastewater treating photobioreactors, other authors have tried using mixtures of different algae species. Carney et al., (2014) used a mixture of *Scenedesmus sp.* and *Desmodesmus sp.* in a proprietary system using wastewater as a growth medium called Offshore Membrane Enclosures for Growing Algae (OMEGA). Using amplicon based sequencing, they were able to demonstrate the inoculation and growth of the algae in the photobioreactor as well as the changes in the bacterial populations.

Others have gone even further and used algae communities with many species in a wastewater treating photobioreactor system (Assemany et al., 2015). Mixed culture systems can be more adaptable to environmental conditions. While the conditions may not always be favourable for a specific monoculture, in mixed culture systems, another algae species may be more adapted to the new conditions and grow dominant. Complex microbial consortia have also been shown to be better adapted to handling and degrading toxins and creating a more stable waste treatment system overall (Muñoz and Guieysse, 2006).

Assemany et al., (2015) described a system based on high rate ponds (HRP) where they were able to identify a total of 32 genera of phytoplankton.

The most abundant class throughout the experiment was *Chlorophyceae,* with *Desmodesmus* being the most dominant genus in the summer and fall, and *Chlorella* in the winter and spring.

Similarly Komolafe et al., 2013 compared two mixed culture wastewater treating photobioreactors to a system inoculated with only *Desmodesmus sp.* While the *Desmodesmus sp.* photobioreactor showed a higher maximum biomass concentration of 0.58 g/L compared to 0.45 g/L, the mixed culture dominated by *Oscillatoria* and *Arthrospira* had a higher lipid and fatty acid methyl ester (FAME) yield.

2.2.2 Bacterial communities

The existence of interactions between microalgae and bacteria in natural environments has been known for decades. In fact the term "phycosphere" was coined more than 40 years ago to describe the area around microalgal cells or colonies "in which bacterial growth is stimulated by extracellular products of the algae" (Bell and Mitchell, 1972).

As wastewater usually contains a wide variety of microorganisms (Ju et al., 2014), a photobioreactor system using wastewater as a growth medium without costly sterilization is bound to contain a rich microbial diversity. While the microbial communities in activated sludge plants have been well documented (Ju et al., 2014), there is a limited number of studies exploring the bacterial communities present in different wastewater treating photobioreactors.

Carney et al., (2014) described the microbiome of a prototype wastewater treating photobioreactor. For this they used an amplicon based method, sequencing the hypervariable region V4 of the eukaryotic small subunit (SSU) rRNA to distinguish algae, and the hypervariable region V6 of the bacterial SSU rRNA to distinguish bacteria. In general, bacteria from the *Proteobacteria* and *Bacteroidetes* phyla were dominant. Immediately after inoculation, *Gammaproteobacteria* such as *Shewanella* and *Rheinheimera* comprised the majority of the bacteria. After 2 weeks however, *Alphaproteobacteria* from the genus *Rhizobium* were dominant.

Similar results were also described by Krohn-Molt et al., (2013) who studied the bacterial biofilm associated with the microalgae *C. vulgaris* and *S. obliquus* in a photobioreactor system using a liquid medium containing fertilizer supplemented with KNO_3. In their analysis, they also reported the dominant phyla to be *Proteobacteria* and *Bacteroidetes.* There were also similarities on lower taxonomic levels. The dominant *Alphaproteobacteria* were from the order *Rhizobiales* and the order *Flavobacteriales* was also found to be well represented in both studies.

Both results are consistent with data from natural environments. Marine macroalgae (of the *Chlorophyta* variety) are known to be associated with

several bacteria from the phyla *Bacteroides* and *Proteobacteria* (Goecke et al., 2010). The same phyla have also been strongly associated with diatoms (Amin et al., 2012).

In agriculture, bacteria are used as inoculants to promote plant growth. The same principle could be applied to microalgae in photobioreactors. Experimenting with algae growth promoting bacteria gained interest in the early 2000s (Gonzalez and Bashan, 2000), and de-Bashan et al., (2004) demonstrated how growing a combination of microalgae (*C.vulgaris* or *C. sorokiniana*) together with *Azospirillum brasilense*, co-immobilized in small alginate beads, in wastewater produced higher growth and better nutrient removal compared to the microalgae without *A. brasilense*.

2.2.3 Interactions between algae and bacteria

There are many interactions between algae and bacteria in both natural and constructed environments. Kouzuma and Watanabe, 2015 categorized them into three types: nutrient exchange, signal transduction and gene transfer (Fig. 4).

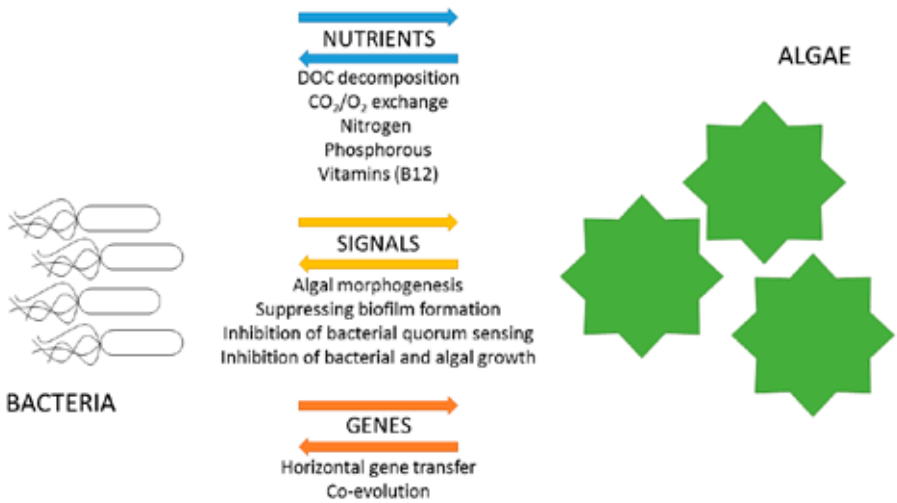

Figure 4. Proposed interactions between algae and bacteria based on Kouzuma and Watanabe, 2015.

Nutrient exchange

Heterotrophic bacteria break down organic matter that is released by algae as dissolved organic carbon (DOC) and release CO_2 that the algae can take up for photosynthesis. The release of CO_2 also helps prevent the pH from rising too high due to the algae reducing carbonate and bicarbonate levels in the water.

The oxygen released through photosynthesis is used by the bacteria to break down organic compounds. In addition, the bacteria can also use the dead algae cells as a source of nutrients (Kouzuma and Watanabe, 2015; Muñoz and Guieysse, 2006). This natural oxidation of water can be valuable for wastewater treatment as aeration is expensive. As the algae release oxygen, there is less need for aeration compared to conventional aerobic wastewater treatment systems, where mechanical aeration can account for 40-60% of the total energy consumption (Shi, 2011). The bacterial consumption of O_2 is also beneficial for algal biomass production, as high O_2 concentrations can inhibit photosynthesis (Christenson and Sims, 2011).

Nitrogen is another important nutrient that is transformed through bacterial and algal interactions. In a recent study, Le Chevanton et al., 2013 suggested that N availability for the microalgae *Dunaliella sp.* was affected by *Alteromonas sp.* and *Muricauda sp.* These bacteria enabled increased N incorporation and enhanced algal growth.

Nitrogen balance in the system can also be affected by the inhibition of certain parts of the consortia. Studies have found that algae and cyanobacteria can inhibit the growth of nitrifying bacteria (Choi et al., 2010). This inevitably has an effect on the nitrogen uptake; Karya et al., (2013) showed that 81-85% of the ammonium in a wastewater photobioreactor was removed through nitrification. This could have an effect on algal growth due to algae generally preferring ammonium over nitrate as a nitrogen source since it is energetically less demanding to import into the cell (Dortch, 1990).

Another example is the synthesis of vitamin B12. Some estimates show that more than half of species in the algal kingdom cannot synthesize their own vitamin B12 and need to acquire it from bacteria (Croft et al., 2005). The bacterial production of B vitamins in photobioreactors was confirmed by genetic and laboratory measurement data by Krohn-Molt et al., (2013). They also found that the bacteria in their reactor encoded many esterolytic and lipolytic enzymes, adding to the research which shows how bacteria consume algal products, which could have an effect on the biofuel production capabilities of a photobioreactor (Kouzuma and Watanabe, 2015).

Signal transduction

Signal exchange between bacteria and eukaryotes such as algae is referred to as inter-kingdom signalling (Hughes and Sperandio, 2008). In signal transduction, chemicals emitted by microorganisms are not used as nutrients but rather as activators or inhibitors of genes and/or physical activities (Kouzuma and Watanabe, 2015).

Bacteria have been shown to induce morphogenesis in algae through chemical signals. A bacterial strain YM2-23 from the Cytophaga-Flavobacterium-Bacteroides group is able to produce a highly potent differentiation inducer called thallusin. This chemical strongly induces the

differentiation of the alga *Monostroma oxyspermum* from loose aggregates of single cells to a leafy morphology (Matsuo et al., 2005).

On the other hand, algae are known to supress the formation of excess biofilms on their surfaces and can inhibit bacterial quorum sensing (Kouzuma and Watanabe, 2015). This suggests co-evolution of algae and bacteria, as these signals are used as defence mechanisms for the algae against algicidal bacteria (Amin et al., 2012).

Another example of these interactions is the removal of pathogens present in wastewater. The microalgae are able to chemically change the environment of the reactor (pH, oxygen concentration) and make it less suitable for pathogenic bacteria (Muñoz and Guieysse, 2006). Ruiz-Marin et al. (2010) demonstrated a 95% reduction of faecal coliform bacteria in a semi-continuously operated bioreactor system with immobilised *S.obliquus* cells. The final concentration was still above the level suitable for discharge so additional treatments may still be necessary. High total coliform removal (up to 99.8%) was also described by (Komolafe et al., 2013) in reactors inoculated with mixed microalgae cultures and with *Desmodesmus sp.*

Gene transfer

Since algae and bacteria live together in phycospheres and interact in complex relationships (Amin et al., 2012), there are many possibilities for horizontal gene transfer. Evidence of such genetic transfer has, for example, been found in the chloroplast genome of *Seminavis robusta* (Brembu et al., 2014). At least two of the plasmid-localised genes of *S. robusta* are thought to be derived from bacteria belonging to the class *Clostridia*.

Recent research into the decay of vitamin-related pathways in eukaryotes, such as pathways related to the synthesis of B-group vitamins (Helliwell et al., 2015) suggests that algae and bacteria have co-evolved, since these vitamins are supplied by the bacteria living near algae (Kouzuma and Watanabe, 2015).

3. Methodology

3.1 Wastewater and lake water origin and properties

The wastewater used in the experiments presented in this thesis was sampled from the inflow of the municipal Wastewater Treatment Plant (WWTP) in the city of Västerås, Sweden. The plant is designed to treat sewage from 118 000 population equivalents. The inflowing raw wastewater is screened, pre-precipitated with iron sulphate and biologically treated with activated sludge process. Glycol is added to the water to support pre-denitrification. Samples were taken from the top layer in the centre of the mixed basin after some of the original phosphorous was removed.

Lake water, used to add algae to the photobioreactor system, was sampled from a yacht harbour near the WWTP from the upper layer (0.5 m) of Lake Mälaren, the third largest lake in Sweden (Kvarnäs, 2001).

Sampling was done following the SS/ISO 5667-3:2004 standard with sterilised equipment and samples were immediately transported to a refrigerator at 4°C.

Specific water sample nutrient concentrations and properties are described in detail in the included publications (Paper I-IV).

3.2 Experimental setup

To test the ability of algae from Lake Mälaren to grow in wastewater an initial proof of concept experiment was set up with 250 mL flasks (Paper I). Lake- and wastewater ratios of 30/70, 50/50 and 70/30 were tested and compared to pure lake- and wastewater samples (Fig. 5a). The flasks were shaken manually every 24 hours and algal growth was measured. The 70/30 wastewater/lake water mixture showed the most growth, and thus this mixture was used in subsequent experiments.

To further test the effect of lake water inoculation, reactors with a volume of 1 L were used (Fig. 5b). Four separate reactors were set up in modified fermenters consisting of glass cylinders with stainless steel tops and bottoms. The reactors were set up with four different conditions:

1. a tap water reactor (TWR) containing 30% tap water and 70% wastewater,
2. a lake water reactor (LWR) containing 30% lake water and 70% wastewater and
3. a wastewater reactor (WWR) containing 100% wastewater.

As a control a sterilized wastewater reactor (SWR) was set up to test for contamination.

The experiments were conducted on three separate occasions with similar conditions to test whether the season when lake water was sampled had an effect on algal growth in the photobioreactor – one in August, one in November and one in December (Paper I).

A similar setup was also used to study the effect of nitrification inhibition on the growth of microalgae (Paper II). The experimental plan was however modified such that all 4 reactors contained 30% lake water pre-grown for 1 week at 23°C and 70% wastewater. The algae were grown in the lake water before the experiment to increase the amount of algae in the water prior to mixing wastewater. Two of the reactors had only WW and LW for normal operation while the other two had 0.05 g of allylthiourea (ATU) added to inhibit nitrification.

The reactor size was scaled up to 20 L and custom photobioreactors were designed and built to study the bacterial and algal community and functional genes in lake water inoculated and uninoculated reactors (Fig. 5c). The reactors were adapted from 3 modified 30 L aquariums (Zolux NanoLife Cube 30) and a stirring mechanism was set up with a plexiglas support to maximize light penetration. Two experiments were conducted, one using three TWRs and another using three LWRs as described earlier.

The specific physical parameters involved in each set up are described in the respective papers but they follow data published by Tang et al., 2011. With each experiment specific limitations had to be accounted for due to the volume or type of reactor used.

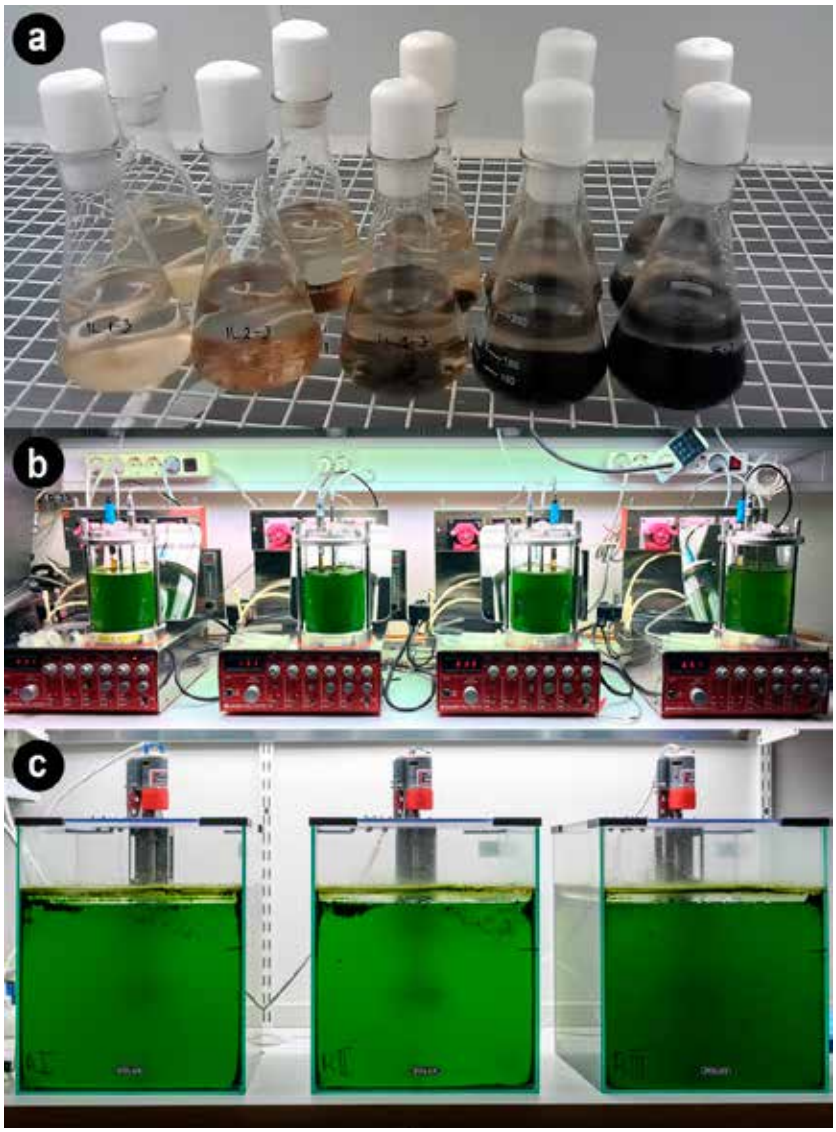

Figure 5. 250 ml flasks used in the proof of concept (Paper I) experiments (a), 1L photobioreactors used in the experiments studying the effect of lake water inoculation (Paper I) and nitrification inhibition (Paper II) on algal growth (b) and 20L photobioreactors designed and built to study the bacterial and algal community and functional genes (Papers III and IV) in lake water inoculated and uninoculated reactors (c).

3.3 Algal community composition and growth dynamics

In the proof of concept experiments conducted in 250 ml flasks to study algal inoculation of wastewater with lake water, optical density (OD) at 630 nm was used as an indicator of algal growth. Due to the complex nature of the wastewater samples this proved to be a poor indicator of true algae growth. As a result subsequent experiments used chlorophyll a concentration measurements to get a better indication of algal growth as described in Bellinger and Sigee, (2010).

The algae present in the reactors were visually examined using an Alphaphot-2 YS2 microscope (Nikon Instruments Inc., Tokyo) at 150x magnification.

3.4 Molecular methods and community analysis

Bacterial analysis presented in this thesis (Papers I and III-IV) was performed using DNA extracted from the water samples. DNA was extracted using MoBio PowerWater DNA extraction kit (Mobio Laboratories Inc., Carlsbad, CA, USA) in all experiments.

The development of the bacterial community (Papers I and III) was estimated using 16S rRNA gene copy numbers. The data was analysed as described by (Nõlvak et al., 2012).

To describe the bacterial and algal communities and their functional genes (Paper III-IV), the metagenomes of the samples were sequenced and analysed.

DNA concentrations were measured with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The samples were diluted in EB buffer (Qiagen, Venlo, Netherlands) and prepared using the Nextera DNA Sample Preparation Kit (Illumina, San Diego, CA, USA). The manufacturer's protocol was modified by using 100 ng input DNA instead of 50ng and the second purification step was replaced with the NucleoSpin kit (Macherey-Nagel, Düren, Germany).

Sample concentrations were measured with the Qubit Fluorometer and samples were normalized. Samples were sequenced with the MiSeq Benchtop Sequencer system (Illumina, San Diego, CA, USA).

The microbial communities present in the sequenced samples were analysed with MG-RAST software (version 3.3.7.3) using SILVA Small Subunit and M5 non-redundant protein (M5NR) databases. Additional community analyses were done with Kraken software (version 0.10.5) (Wood and Salzberg, 2014). The MG-RAST M5NR database and the human pathogenic bacteria virulence factor database (VFDB) were used to detect the presence of pathogenic bacteria. The VFDB was probed with the Lambda Local Aligner for Massive Biological Data (version 0.4.7) (Hauswedell et al., 2014).

Longer reads known as contigs were assembled from the sequences using Megahit software (version 0.3.2) (Li et al., 2015) and their quality was tested with Quast (version 2.3) (Gurevich et al., 2013).

The abundance of nitrogen metabolism and antibiotic resistance genes was estimated using HMMER3 to search FOAM (Functional Ontology Assignments for Metagenomes) functional gene database (Prestat et al., 2014) and Resfams curated database of protein families and associated profile hidden Markov models (HMMs) confirmed for antibiotic resistance function (Gibson et al., 2014) respectively.

3.5 Nutrient and metal removal

Nutrient concentrations were measured at the beginning and end of the experiments studying the effect of inoculation and nitrification inhibition on algal growth and the algal and bacterial communities. In most cases, nutrient dynamics over the experimental period were also measured. The nutrients under investigation were TOC, dissolved organic carbon (DOC), ammonium (NH_4), nitrate (NO_3) and total phosphorous (TP), as these are important for the growth of microorganisms and their removal by the system is an important goal.

In order to understand nitrogen uptake by algae, nitrification was inhibited in some experiments and compared to samples with functional nitrification (Paper II). This changed the nitrogen speciation in the wastewater. When nitrification was inhibited, most of the nitrogen was in the form of ammonium while in the control reactors the nitrifying bacteria in the wastewater produced a mixture of ammonium and nitrate.

Metal concentrations were also measured at the beginning and end of the experiments when studying the effect of lake water inoculation (Papers I and III). The focus was on Cr, Co, Ni, Cu, Zn, As and Cd as these were the main metals of interest in the wastewater used and for the performance of an algal system.

3.6 Statistical analysis

Descriptive statistics were used throughout the experiments. Where relevant, common statistical methods such as averages, standard deviations and confidence intervals were calculated using Excel (Microsoft). Two-way Analysis of Variance (ANOVA) was used to determine significance when comparing the treatments.

In order to study the effect of inoculation on the different functional gene groups, principal component analysis (PCA) was performed on the metagenome data from inoculated and control reactors (Paper III). The data was normalized using arcsine square root transformation. PCA can represent as much of the variation as possible using only a few axes, allowing for many

variables to be considered together. In this case, the functional gene groups in several samples could all be compared simultaneously.

In order to validate the significance of differences in the presented metagenome data (Papers III and IV), STAMP (Statistical Analysis of Metagenomic Profiles) v 2.0.2 software was used to analyse the differences in community and functional gene groups in different metagenomic profiles extracted from the samples (Parks and Beiko, 2010).

4 Results and Discussion

4.1 Photobioreactor performance and algal growth

Overall we were able to get algal growth in both photobioreactor systems used – the 1L modified fermenters and the 20 L modified aquariums (Fig. 6-7). This shows that the design parameters chosen for light, stirring and gas exchange were suitable for growth.

The 1L reactors used to analyse the effect of inoculation on algal growth showed an increase in chlorophyll a concentration in all reactor set-ups: lake water reactors, tap water reactors and wastewater reactors. There were however differences in the maximum growth and growth rate depending on whether the reactors were inoculated, diluted or only contained wastewater, and on the season during which the inoculant was collected (Paper I). Not surprisingly, the algal growth rate was fastest and overall maximum growth was highest in the reactors inoculated with lake water sampled during summer. This is most likely due to the higher concentration and variety of active algae and cyanobacteria present in the lake water at that time. It is also likely that the lab conditions with respect to light and temperature were favourable for the dominant organisms present at that time. While both the lake water and tap water reactors reached a comparable maximum chlorophyll a concentration, the growth rate in the reactor with lake water was 2-3 days ahead of the others. This shows that the inoculation had an effect on the algal growth and that the difference was not just due to the dilution of the wastewater medium.

When the reactors were inoculated with lake water sampled during the colder autumn season, algae growth was considerably slower compared to the reactors inoculated with samples collected in summer. The maximum chlorophyll a concentration in all the reactors was around half of what it was when the lake water inoculant was sampled during the summer. However, the reactors inoculated with lake water still had higher maximum chlorophyll a concentrations and growth rates.

Algal growth was lower still when reactors were inoculated with lake water collected when the lake surface had frozen and this lake water had no noticeable effect on growth. This may be due to the low temperature of the lake water and the algae being dormant. It is also possible that the algae that were in the lake water were not suited to growth in the relatively warm

conditions of the photobioreactor and would have performed better if more time was allowed for adaptation.

Algal growth was also compared between reactors inoculated with lake water and those diluted with tap water in larger 20 L reactors (Paper III). There was a considerable difference in the chlorophyll a concentration between the two experiments on the 16th day. The lake water reactors showed significantly more algae growth ($p<0.001$, $n=3$) than the tap water reactors, having on average 38% higher maximum chlorophyll a concentrations. The inoculated reactors also showed a higher growth rate, and reached peak chlorophyll a concentrations at 12 days, after which they began to stabilize (Fig. 6). The tap water reactors, however showed less variation between triplicates, resulting in a lower standard deviation. This could be due to the difficulty in achieving consistent inoculations between the replicates.

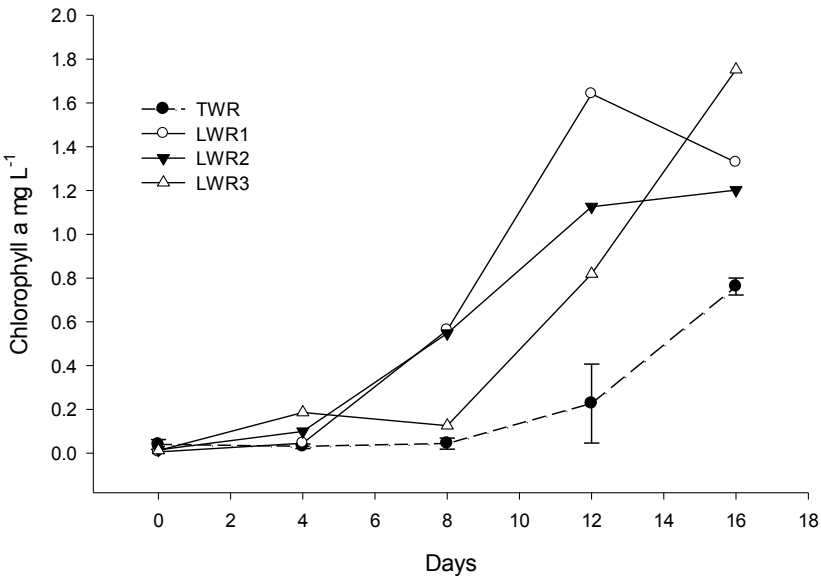

Figure 6. Dynamics of chlorophyll a during the experimental period. Shown are arithmetic means of the triplicate treatments in the tap water reactors (TWR), lake water reactors (LWR) and separate values for each lake water reactor (LWR 1-3) where needed due to the large differences between LWR1-2 and LWR3. Error bars indicate confidence intervals.

The effect of nitrification inhibition on algal growth in the photobioreactors was also studied (Paper II). The experiments showed that the algae were able to grow to a higher maximum chlorophyll a concentration when nitrification was inhibited (Fig. 7). The difference between the inhibited and the control samples was not apparent during the first 4 days of the experiment, however the difference became more apparent as more NH_4 was nitrified (Fig. 7). This was most likely to be because algae prefer ammonium over nitrate (Dortch, 1990). It is less energetically demanding to import NH_4 into the cells and in this experiment, this translated into a higher algal growth.

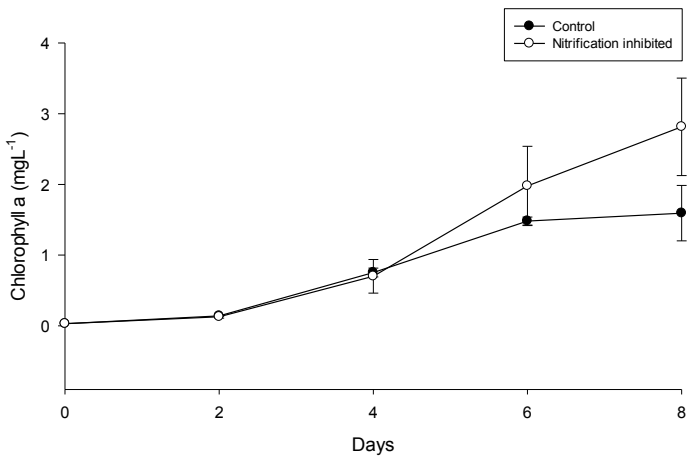

Figure 7. Dynamics of chlorophyll a concentrations in the control and nitrification inhibited reactors. Error bars indicate standard deviation.

4.2 Nutrient dynamics and metal removal

4.2.1 Carbon

Carbon concentrations were measured before and after the photobioreactor experiments to estimate how much carbon the algae had taken up. As the algae took up CO_2, Total Organic Carbon (TOC) concentrations increased in all the reactors. DOC showed a concomitant decrease, suggesting that the algae had taken up the carbon from the water phase. There was a larger decrease in DOC concentrations and a larger increase in TOC concentrations in the reactors that were inoculated with lake water. This reflected the fact that these reactors had the highest algal growth. Overall the TOC concentrations increased around 2 fold and the DOC in the wastewater was reduced by around 50-60%.

4.2.2 Nitrogen and phosphorous

As with carbon, nitrogen and phosphorous were taken up in relation to the algal growth in the reactors. Nitrogen was mostly introduced into the reactors

in the form of ammonium from the wastewater. The initial nitrogen concentrations were on average 30-40 mg L^{-1}. During the experiments, the ammonium concentration decreased and the NO_3-N concentration increased. The increase in nitrate concentration is most likely due to the high concentration of nitrifying bacteria commonly found in wastewater (Harms et al., 2003). Because the reactors are aerobic, the bacteria can quickly nitrify the ammonium before the algae start to grow. Similar results have been reported by Karya et al., (2013) who showed that 81-85% of the ammonium in a wastewater photobioreactor was removed by nitrification and not through uptake by algae.

In experiments conducted to test the effect of lake water inoculation, >99.9% of the ammonium was removed after 4 days (Paper III). NO_3-N concentration increased compared to the initial concentration but stayed below 0.7 mg L^{-1} in both the lake water inoculated and tap water diluted reactors, showing that the nitrate resulting from nitrification was quickly assimilated by the algae.

This purification efficiency is in agreement with wastewater treating photobioreactor studies by other groups. For example Di Termini et al., (2011) reported a 90-99% reduction in ammonium concentration and Riaño et al., (2012) achieved a reduction of more than 99%.

Because nitrification plays such an important role in wastewater treating photobioreactors (Karya et al., 2013), it was chemically inhibited and compared to uninhibited control reactors to study the effect on the speciation and concentration of nitrogen (Paper II). As expected the NH_4-N concentration in the control reactors decreased much more rapidly than in the nitrification inhibited reactors (Fig. 8a). By day 5 the NH_4-N concentration in the control reactors had reached below 0.05 mg L^{-1}, and while they increased after additional wastewater was added, the NH_4-N was nitrified by the next day. By that time potential nitrification (Fig. 8c) had also reached a maximum level of 19.7±1.7 mg N 10 mL^{-1} day^{-1}. By contrast, the NH_4-N concentration decreased slowly over the cultivation period in the nitrification inhibited reactors resulting in a final concentration of 13.3±5.8 mg L^{-1}.

The effect of nitrification was also apparent in the sum NO_3-N+NO_2-N concentrations (Fig. 8b), which started increasing after day 2 in the control reactors while decreasing in the nitrification inhibited reactors. This further indicated that nitrification was the cause of the rapid NH_4-N decrease in the control reactors. Nitrification had a strong effect on the nitrogen speciation in the reactors. While the final NH_4-N+NO_3-N+NO_2-N concentration in the control and inhibited reactors was very similar after 8 days of algae cultivation, the nitrogen speciation was different, with N existing mostly as NH_4-N in the inhibited reactors and as NO_3-N in the control reactors. Because it is less energetically demanding for algae to import NH_4 into their cells (Dortch, 1990), this had an effect on algal growth, with higher growth reported in the inhibited reactors.

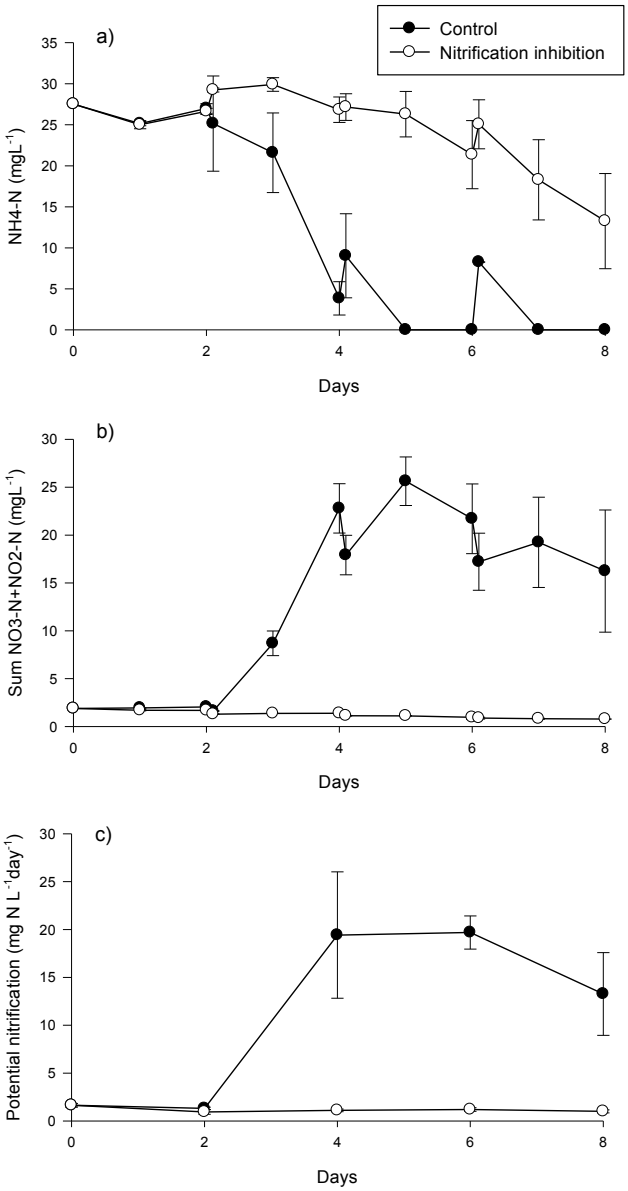

Figure 8. Dynamics of NH_4-N concentration (a) sum NO_3-N+NO_2-N concentration (b) and potential nitrification (c) in the control and nitrification inhibited reactors. Error bars indicate standard deviation.

Total phosphorous concentrations in the incoming water were 1-4 mg L^{-1} in all the experiments. However, not all of the phosphorus was readily available to the algae and as in the experiments with nitrification inhibition,

dissolved phosphorous concentrations were low, remaining below 0.5 mg L^{-1} throughout (Paper II). This is mostly due to the way wastewater is handled in the plant. Much of the phosphorous has already been removed when it enters the treatment process. Because of these low initial concentrations, the algae were able to reduce dissolved P concentrations to below 0.05 mg L^{-1} by the end of the experimental period in all tested conditions.

4.2.3 Metals

To determine the change in metal concentrations, Cr, Co, Ni, Cu, Zn, As, Cd and Pb concentrations were analysed (Paper III). These metals were selected for analysis due to their prevalence in the wastewater used and the number of reports in the literature on their removal with algae.

Co and Zn concentration were significantly ($p<0.001$) reduced both in the tap water and lake water reactors without significant differences between the conditions. The average reduction of Co was 75.6±2.2% in the tap water diluted reactors and 56.5±11.7% in the lake water inoculated reactors. For Zn the respective reductions were 63.6±22.7% and 82.1±3.9%. It is likely that these reductions were due to microorganisms like *C. vulgaris* and *S. Obliquus,* which were present in both reactor types and are known to remove Zn, Cr, Cu and Ni from the water phase (Çetinkaya Dönmez et al., 1999; Travieso et al., 1999). However, Cr, Ni and Cu concentrations showed a significant increase in tap water reactors ($p<0.01$, $p<0.05$ and $p<0.01$ respectively), while in the inoculated reactors Cr and Cu showed no significant change and Ni showed a statistically significant reduction ($p<0.05$). It is possible that other microorganisms present in the reactors were mobilizing some of the metals while the algae were adsorbing them (Gadd, 2004). As, Cd and Pb showed no statistically significant changes in either reactor system.

4.3 Algae community composition

Algal community was analysed microscopically for experiments performed in the 1L photobioreactors (Papers I and II) and by metagenome analysis for experiments performed in 20L photobioreactors (Papers III and IV).

As can be seen by the heatmap (Fig. 9), most eukaryotes in the reactors belonged to the phylum *Chlorophyta*, which includes green algae. This was especially true in the reactors that were inoculated with lake water, where the general algae abundance was considerably higher than in the tap water reactors. Compared to the wastewater samples their concentration was increased significantly. At the same time, the relative abundance of *Ascomycota* (fungi) and *Arthropoda* genes was lower in the reactors with added lake water (Fig. 9).

Overall the dominant genera found in the photobioreactor systems studied in this thesis were: *Scenedesmus, Desmodesmus* and *Chlorella* (Papers I-IV).

The metagenome analysis (Papers III and IV) provided information about the dominant algae at the species level and revealed differences between the reactors that were inoculated with lake water and the tap water reactors (Table 1). In the tap water reactors, the most abundant algae species were *Chlorella vulgaris, Nitzschia frustulum, Phaeodactylum tricornutum, Gomphonema affine* and *Micractinium pusillum*. However, the lake water reactors were dominated by *Scenedesmus obliquus, Desmodesmus costato-granulatus, Scenedesmus acutus, Chlorella vulgaris* and *Pseudopediastrum kawraiskyi*, with *Scenedesmus obliquus* being the most abundant in all replicates. As well as the differences in the dominant species, the relative abundances of the algae differed substantially between the lake water and tap water reactors (Table 1).

Wastewater treating photobioreactor systems with *Scenedasmus, Desmodesmus, Chlorella* as the dominant algal species are common in the literature (Carney et al., 2014; Wu et al., 2014). *Scenedesmus* and *Chlorella* species are also very adaptable to growth in wastewater and are able to grow photoautotrophically and mixotrophically depending on the characteristics of the wastewater used (Wu et al., 2014). These adaptations are most likely the reason that they became dominant in the reactor systems tested.

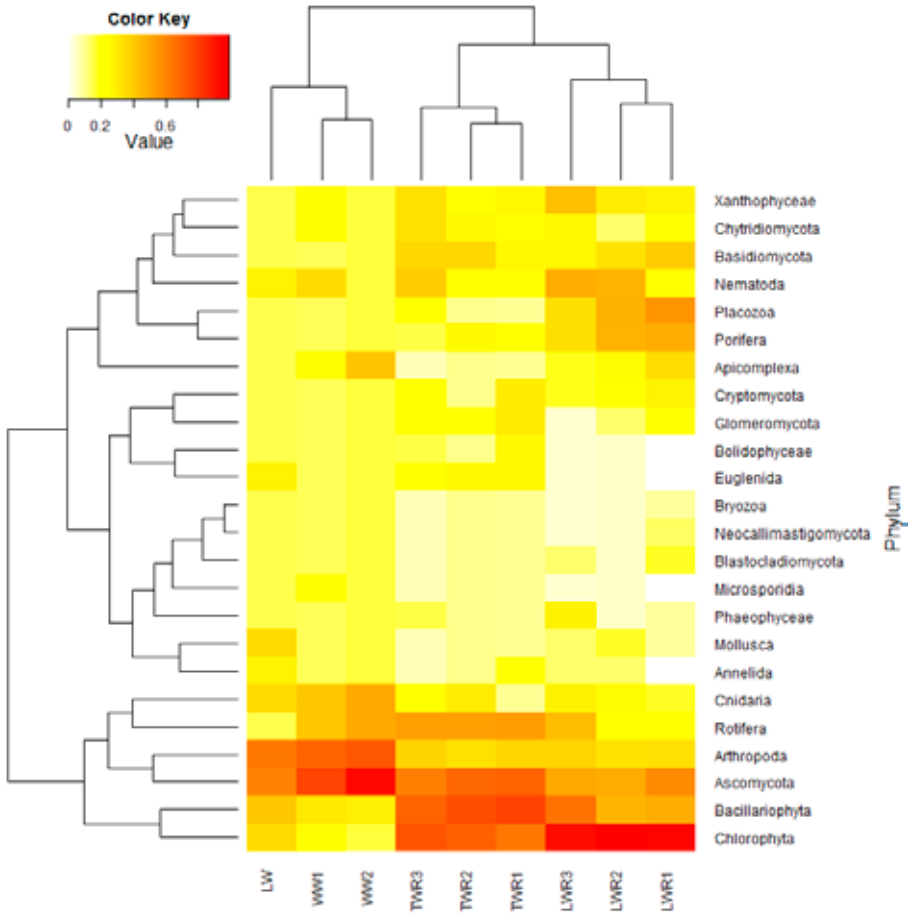

Figure 9. Heat maps based on the rRNA reads annotated using SILVA SSU database of eukaryote phyla in the lake water (LW), wastewater (WW1, WW2), tap water reactor (TWR1-3) and lake water reactor (LWR1-3) samples. Colour intensity (white to red) shows relative abundance of the specific phyla in the sample groups.

Table 1. The five most abundant algae species (by average number of hits in the metagenome) in the tap water reactors (TWR1-3) and lake water reactors (LWR1-3) based on the rRNA reads annotated using the SILVA SSU database of bacterial ribosomal RNA.

No	Species	TWR 1	TWR 2	TWR 3	Average
1	*Chlorella vulgaris*	5	40	113	53
2	*Nitzschia frustulum*	17	22	33	24
3	*Phaeodactylum tricornutum*	16	8	18	14
4	*Gomphonema affine*	14	10	15	13
5	*Micractinium pusillum*	1	5	11	6
No	Species	LWR 1	LWR 2	LWR 3	Average
1	*Scenedesmus obliquus*	916	1984	632	1177
2	*Desmodesmus costato-granulatus*	200	100	17	106
3	*Scenedesmus acutus*	86	160	63	103
4	*Chlorella vulgaris*	51	148	101	100
5	*Pseudopediastrum kawraiskyi*	52	153	76	94

4.4 Bacterial abundance and community dynamics

Bacterial abundance was estimated by quantifying the 16S rRNA gene in experiments designed to determine the effect of lake water inoculation (Paper I and III).

In general, there was a significant decline ($p<0.001$) in bacterial 16S rRNA gene abundance, indicating a decline in bacterial populations (Fig. 10). The final values were on average 2.8 and 2.5 times lower compared to the respective initial values (Paper III). The decline was most apparent during the first 8 days of the reactor run, after which the abundance stabilized. This was seen in all experiments, regardless of whether they were inoculated or not. The only exceptions were the 1L reactors inoculated with water sampled during summer (Paper I). In this case there was no decline and the bacterial 16S rRNA gene abundances were relatively stable throughout the experiment. This may be due to sampling errors or other unknown factors that made this data point an outlier.

Statistical analyses revealed a strong negative correlation (Spearman $R=-0.87$; $p<0.001$) between 16S rRNA gene copy numbers and chlorophyll a concentrations in the lake water inoculated reactors (Paper III). While there was a similar trend in the tap water reactors, it was not statistically significant

($p>0.05$), most likely due to slower algal growth compared to the lake water reactors.

The overall decrease and eventual stabilization of the bacterial communities as the algae are growing indicates competition between algae and bacteria for the available nutrients and ecological niches (Amin et al., 2012). There may also be a selective pressure from the growth of algae that limits certain bacteria present in the original wastewater community. For example Choi et al., (2010) found evidence that algae and cyanobacteria can inhibit nitrifying bacteria growth in a bioreactor by a factor of 4. In addition, the pH of the system increases with time, and there may be selective pressure in the photobioreactor that selects for bacteria better suited to the emerging algal community.

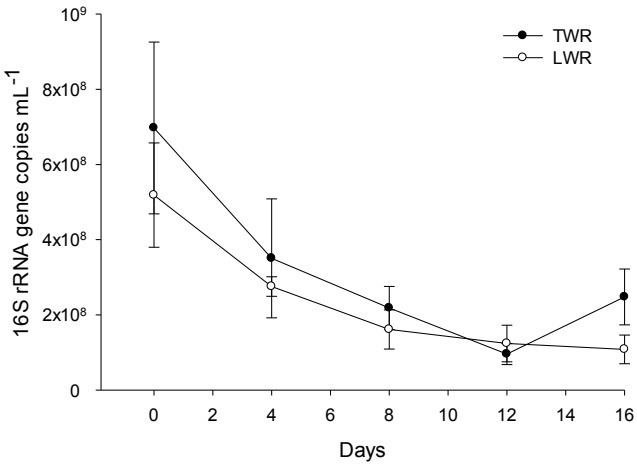

Figure 10. Dynamics of 16S rRNA gene copy numbers in the lake water inoculated reactors and tap water reactors (Paper III). Abbreviations: LWR – Lake water reactor with 70% wastewater and 30% lake water; TWR – Tap water reactor with 70% wastewater and 30% tap water.

As well as using the overall bacterial abundance, the community composition was analysed through metagenomics analysis (Papers III and IV). The most abundant bacteria in the studied systems belonged to the phyla *Proteobacteria* and *Bacteroidetes* with the most dominant families in both treatments being *Sphingobacteriaceae, Cytophagaceae, Flavobacteriaceae, Comamona-daceae, Planctomycetaceae, Nocardiaceae* and *Nostocaceae.*

Proteobacteria and *Bacteroidetes* are both commonly found near algae and are known for interacting with both macro- and microalgae (Amin et al., 2012; Goecke et al., 2010; Le Chevanton et al., 2013). For instance, bacteria from these phyla have been identified as promoting algal growth (Le Chevanton et

al., 2013) and inducing algal morphogenesis (Matsuo et al., 2005). These bacterial phyla were also observed by Krohn-Molt et al. (2013) and Carney et al. (2014), which suggests that they may be a common component in the bacterial composition living with algae in mixed community photobioreactors.

Compared to initial wastewater, *Actinobacteria* and *Firmicutes* abundance decreased in both tap water and lake water reactors (Fig. 11) indicating that they were selected out by the growing algae. There were also changes in the dominant families under *Actinobacteria* and *Firmicutes*.

There were also differences between the most dominant families overall (Table 2). In the wastewater samples before treatment, *Comamonadaceae, Rhodocyclaceae, Burkholderiaceae* and *Bacteroidaceae* were dominant. After 16 days the dominant families in tap water reactors were similar to the wastewater samples, with *Comamonadaceae* still being the most dominant followed by *unclassified Burkholderiales, Sphingobacteriaceae* and *Planctomycetaceae*. The first two replicates of the lake water inoculated reactors differed significantly from wastewater samples with *Rhodobacteraceae*, *Erythrobacteraceae* and *Sphingomonadaceae* being the dominant bacterial families. Because *Rhodobacteraceae* includes gram-negative chemoorganotrophs and photoheterotrophs capable of synthesizing vitamin B12, which is an important metabolite for many algae (Croft et al., 2005), these results suggest that they have been selected for as a result of cooperation with the algae. The dominant species found in the reactors was *Rhodobacter sphaeroides*, which is a metabolically highly versatile bacterium. It can grow by either aerobic or anaerobic respiration, photosynthesis or fermentation. It is also able to grow under high O_2 tension (Mackenzie et al., 2007), making it a good candidate for algal-bacterial commensalism or symbiosis. As these bacteria did not dominate in the tap water reactors and pure wastewater and lake water samples, we can hypothesize that the community in lake water inoculated reactors was more beneficial for the algae. The bacterial community in the third lake water reactor was somewhere in between pure wastewater samples and the other two replicates with the dominant families being *Comamonadaceae, Rhodobacteraceae* and *Burkholderiaceae*. This may be due to the lower algal growth in the third replicate and issues that may have arisen during inoculation.

Phototrophic bacteria from *Cyanobacteria* and *Chloroflexi* phyla were also detected in the reactors. *Chloroflexi* phyla contained only one identified bacterium, *Herpetosiphon aurantiacus*, which is not photosynthetic but is capable of predation of other bacteria (Quinn and Skerman, 1980). Cyanobacteria were also not diverse in the studied reactors. *Dolichospermum macrosporum* was identified as the most abundant cyanobacteria. However, the third replicate of the LWRs diverged from its replicates in having a more diverse community of cyanobacteria with the most abundant cyanobacteria belonging to the genus *Pseudanabaena*.

Table 2. The ten most abundant bacterial families (by average proportion) in the tap water reactors (TWR) and lake water reactors (LWR) based on the rRNA reads annotated using the M5 non-redundant protein database (M5NR) in MG-RAST. Proportions in the wastewater (WW) and lake water (LW) used are shown for comparison.

No.	Family	TWR	WW	LW
1	*Comamonadaceae*	0.10±0.06	0.18	-
2	*unclassified Burkholderiales*	0.06±0.01	0.04	-
3	*Sphingobacteriaceae*	0.04±0.01	0.01	-
4	*Planctomycetaceae*	0.04±0.01	0.004	-
5	*Flavobacteriaceae*	0.03±0.01	0.03	-
6	*Burkholderiaceae*	0.03±0.01	0.04	-
7	*Cytophagaceae*	0.03±0.01	0.01	-
8	*Rhodobacteraceae*	0.03±0.004	0.02	-
9	*Sphingomonadaceae*	0.3±0.02	0.01	-
10	*Xanthomonadaceae*	0.02±0.002	0.01	-
No.	Family	LWR	WW	LW
1	*Rhodobacteraceae*	0.22±0.15	0.01	0.01
2	*Sphingomonadaceae*	0.08±0.05	0.003	0.01
3	*Comamonadaceae*	0.07±0.07	0.20	0.23
4	*Erythrobacteraceae*	0.06±0.05	0.001	0.002
5	*Cytophagaceae*	0.03±0.02	0.005	0.01
6	*Acetobacteraceae*	0.03±0.02	0.002	0.004
7	*Flavobacteriaceae*	0.03±0.01	0.02	0.06
8	*Rhizobiaceae*	0.03±0.01	0.01	0.01
9	*Planctomycetaceae*	0.02±0.02	0.002	0.004
10	*Bradyrhizobiaceae*	0.02±0.003	0.01	0.01

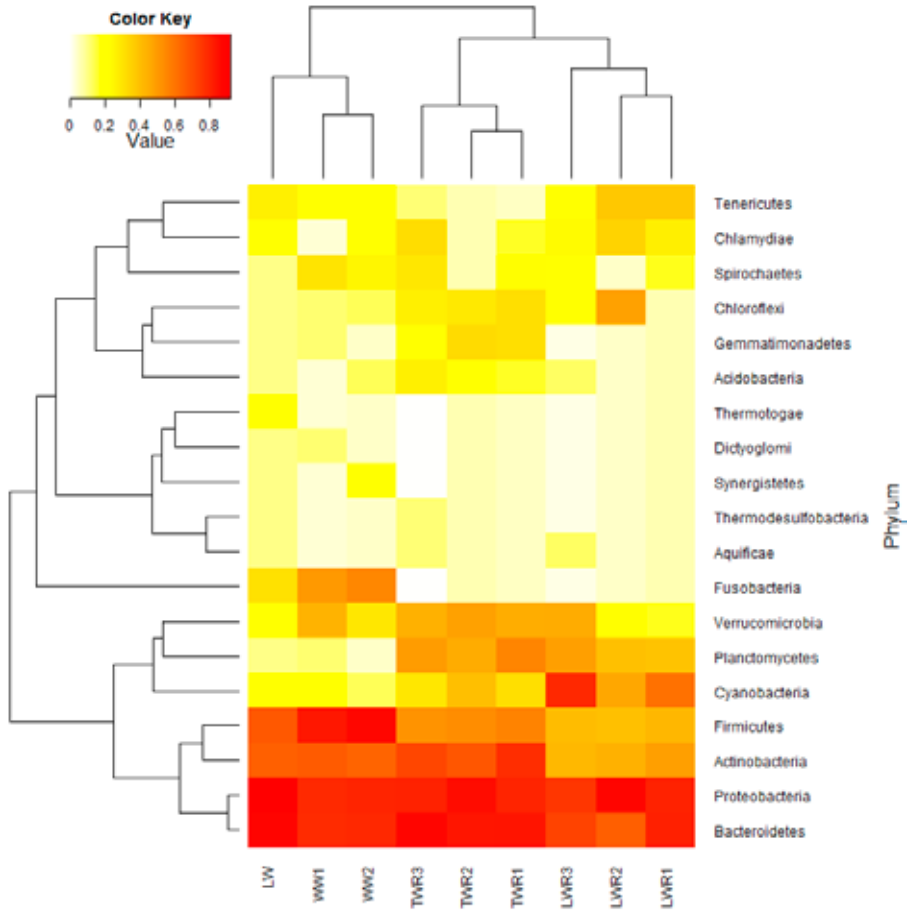

Figure 11. Heat maps based on the rRNA reads annotated using SILVA SSU database of prokaryote phyla in the lake water (LW), wastewater (WW1, WW2), tap water reactor (TWR1-3) and lake water reactor (LWR1-3) samples. Colour intensity (white to red) shows relative abundance of the specific phyla in the sample groups.

4.4.1 Pathogen removal

The reactor samples were also examined for the presence and relative abundance of pathogens (Papers III and IV). Because wastewater can contain several human pathogens, it is important for any wastewater treating system to remove them. In total 36 different human pathogens listed in the virulence factor database (VFDB) were detected in the studied samples (Fig. 12). Overall, both treatments successfully reduced the overall abundance of pathogens in the wastewater. In tap water reactors the relative abundance of pathogens decreased less than in reactors inoculated with lake water (from 0.0151 to 0.0123±0.0005 and from 0.029 to 0.0085±0.0027 respectively) during the 16 day incubation. The relative abundance of pathogens belonging to *Clostridium, Streptococcus, Listeria* and *Shigella* genera was particularly reduced in the lake water reactors. This may be due to the difference in the algal and bacterial communities where pathogens were outcompeted; however, the pH was considerably higher in the lake water reactors, and this is also likely to have had an effect on the survival of the pathogen population. In the case of algal ponds, high pH is one of the mechanisms contributing to faecal bacteria removal (Awuah, 2006; Posadas et al., 2015).

Additional statistical analysis of the samples found that while there was a reduction in pathogen abundance in the reactor systems compared to the initial wastewater (Fig. 12), the differences between the wastewater, lake water and tap water reactor samples were not statistically significant. Because metagenomics analysis can only give results based on the DNA present and says nothing about the viability of bacterial cells, other methods more commonly used for the detection of human pathogens have to be used to further the understanding of pathogen fate in photobioreactors.

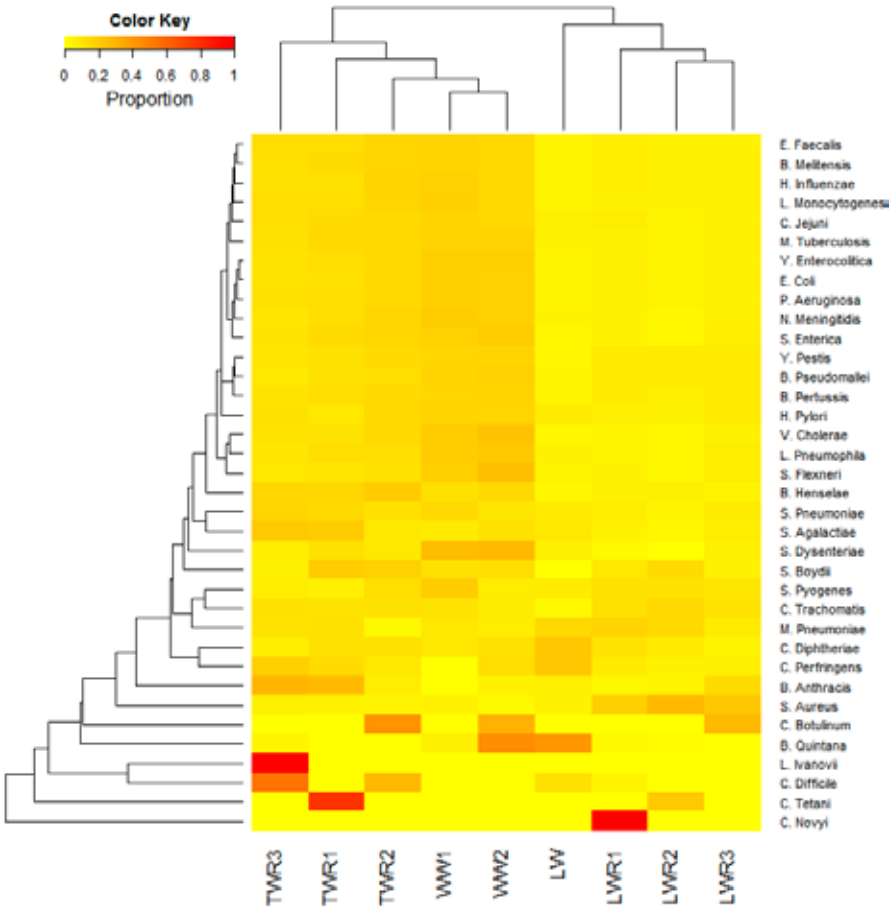

Figure 12. Heatmap of pathogenic bacteria found by analyzing the sequences of the wastewater (WW1-2), lake water (LW), tap water reactor (TWR1-3) and lake water reactor samples (LWR1-3) for markers contained in the VFDB.

4.5 Analysing the function of the genes present in the metagenomes

In order to understand the metabolic pathways present in the metagenome, the functions of the detected genes were analysed (Papers III and IV). Initial analysis was performed based on 28 different subsystems of the SEED project (Overbeek et al., 2005) to get an overview of which functional genes were present in the photobioreactors (Paper III). In the inflow samples, there were several functional differences between lake- and wastewater communities. The communities present in lake water had a higher abundance of genes related to phages, prophages, transposable elements and plasmids, and more importantly in photosynthesis subsystems. This indicates that there were relatively more photosynthetic organisms present in the lake water samples which made their way into the inoculated reactors. On the other hand, the wastewater samples were higher in virulence, motility, chemotaxis and nitrogen metabolism subsystems compared to the other samples. These genes were therefore also present when the photobioreactor system was started up, contributing to the overall nitrogen metabolism. This also means that the pathogens that have the virulence genes still need to be reduced further to meet water treatment standards.

After treatment the gene subsystem profiles looked very different. The genes that were related to nitrogen metabolism and virulence had decreased in relative abundance while genes in subsystems related to photosynthesis, vitamins, cofactors, prosthetic groups and pigments had increased. This was especially true in the lake water inoculated reactors that had the highest number of genes related to photosynthesis and vitamin production. The reactors had a high proportion of B-group vitamins, particularly pyridoxine, biotine and folate related genes, which most likely served the algae that require these vitamins (Croft et al., 2005). There was also a difference in the bacterial populations that had the ability to synthesize vitamin B12. The most dominant bacteria containing the genes needed for vitamin B12 synthesis in the tap water reactors could not be identified by MG-RAST. The second and third highest gene abundance were from *Methylibium petroleiphilum* and *Leptothrix cholodnii*, both members of the *Burkholderiales* order. In the lake water inoculated reactors, it was *R. sphaeroides* from the *Rhodobacterales* order which contributed the most cobalamin and coenzyme B12 synthesis genes. This means that there was a distinct difference in the bacteria providing B12 vitamins to the algae. It is possible that this also had an effect on the growth rate of the algae.

These differences between the genes in the different subsystems is also seen in the PCA presented in Fig. 13. There is a clear development along the PC-2 axis from pure wastewater samples towards the community in the photobioreactor samples. The samples with the highest chlorophyll a content and thus the most algae are furthest away from the wastewater samples.

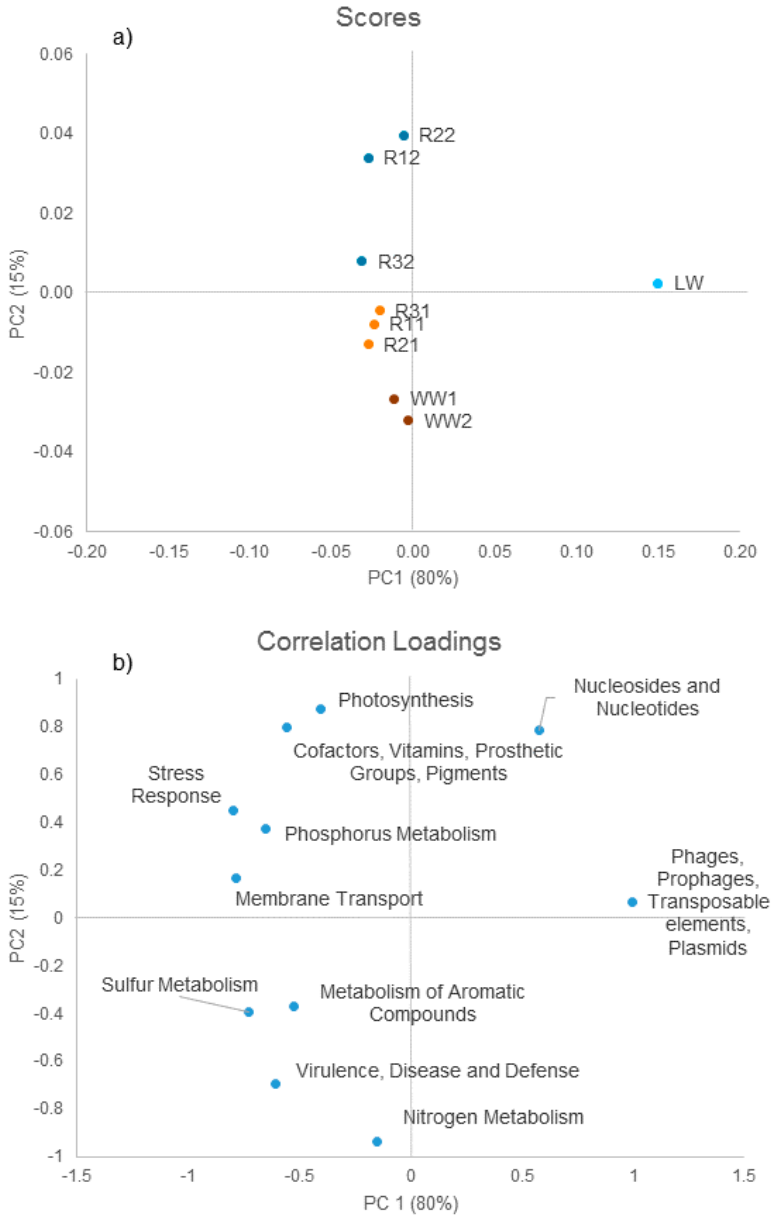

Figure 13. Correlation (a) and loading plots (b) of a principal component analysis based on the relative abundance of the SEED subsystems for the metagenomes of the lake water (LW), wastewater (WW1, WW2), tap water reactor (TWR1, TWR2, TWR3) and lake water reactor (LWR1, LWR2, LWR3) samples. Principal component axes 1 and 2 (a and b) and 2 and 3 (c and d) are shown.

4.5.1 Nitrogen transformations

As the PCA of the metabolic subsystems suggests (Fig. 13), the overall nitrogen metabolism genes were less abundant in the lake water reactors compared to wastewater and tap water reactor samples (Fig. 14) (Paper IV). Nitrification gene abundance was significantly lower ($p<0.05$) compared to wastewater in both tap water and lake water samples. It is possible that the abundance of nitrifying organisms decreases as the ammonium is consumed. Additionally, the growth of microalgae and cyanobacteria has been found to inhibit the growth of nitrifying bacteria in photobioreactors (Choi et al., 2010).

Compared to the lake water reactors and the inflowing wastewater samples, the tap water reactors had a significantly higher ($p<0.05$) abundance of genes involved in dissimilatory nitrate reduction. Genes for NrfB, C and D, which take part in the reduction of nitrate to ammonia, were found. This is evidence that some of the nitrate in the system may be reduced back to ammonium, which is more efficiently assimilated by the algae (Dortch, 1990). These genes may become less important as more of the nutrients are taken up, which could explain why they were less abundant in the lake water inoculated reactors, which had a higher abundance of algae.

If the third replicate of the lake water inoculated reactor, which was significantly different from the other two replicates, was removed from the analysis, there were also significant ($p<0.05$) differences between the lake water and tap water reactors in abundances of genes involved in ammonia assimilation, denitrification and nitrification, the first of which was higher in lake water reactors while the other two were higher in tap water reactors. This agrees with the PCA (Fig. 13) in that the lake water reactors generally had a lower abundance of genes related to nitrogen metabolism.

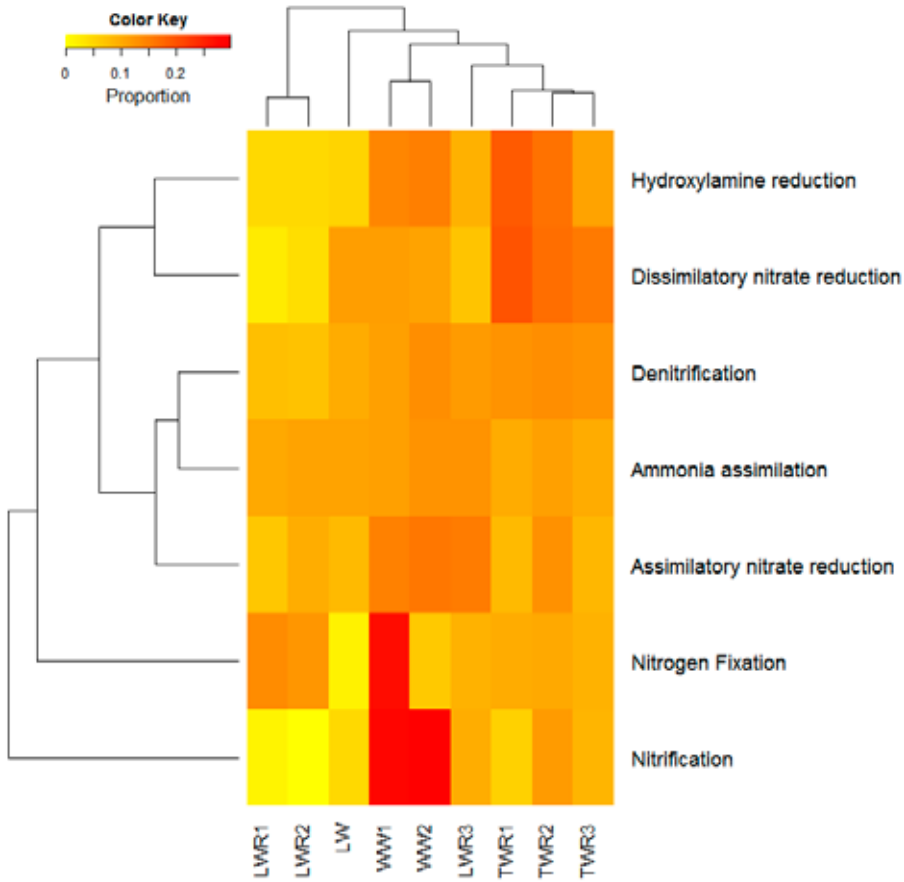

Figure 14. Heat maps indicating differences in relative abundances of functional genes involved in the nitrogen cycle in the metagenomes of the wastewater (WW1-2), lake water (LW), tap water reactor (TWR1-3) and lake water reactor samples (LWR1-3).

4.5.2 Antibiotic resistance

Since wastewater treatment plants are not always effective in removing antibiotic resistance genes (ARG) and can sometimes even increase their concentration in the effluent, the fate of these genes in the photobioreactors was analysed (Paper IV).

The overall relative abundance of ARGs was lower in the lake water inoculated reactors compared to the inflow (Fig. 15), however these differences were not found to be statistically significant. The tap water reactors were not as successful in reducing the abundance of ARGs in the wastewater as the lake water inoculated reactors. This may be due to the relatively lower algal growth and pH values recorded in these experiments. However, some statistically significant differences were found when comparing tap water reactors to the inflow wastewater samples. The tap water

reactors had a significantly lower ($p<0.05$) relative abundance of Target Redundancy/Overexpression genes and a significantly higher ($p<0.05$) relative abundance of Other genes.

Because of the constraints of the metagenomics analysis, the dependence on the extracted DNA and only showing gene abundance and not expression, it is difficult to draw firm conclusions regarding the fate of ARGs. The metagenome data suggests that the genes that play a role in antibiotic resistance mechanisms are reduced in abundance in the reactors in parallel with the pathogenic bacteria but this is an area of photobioreactor research that has not yet been properly investigated, and more detailed analysis is needed to increase understanding of the issue of ARG fate in photobioreactors.

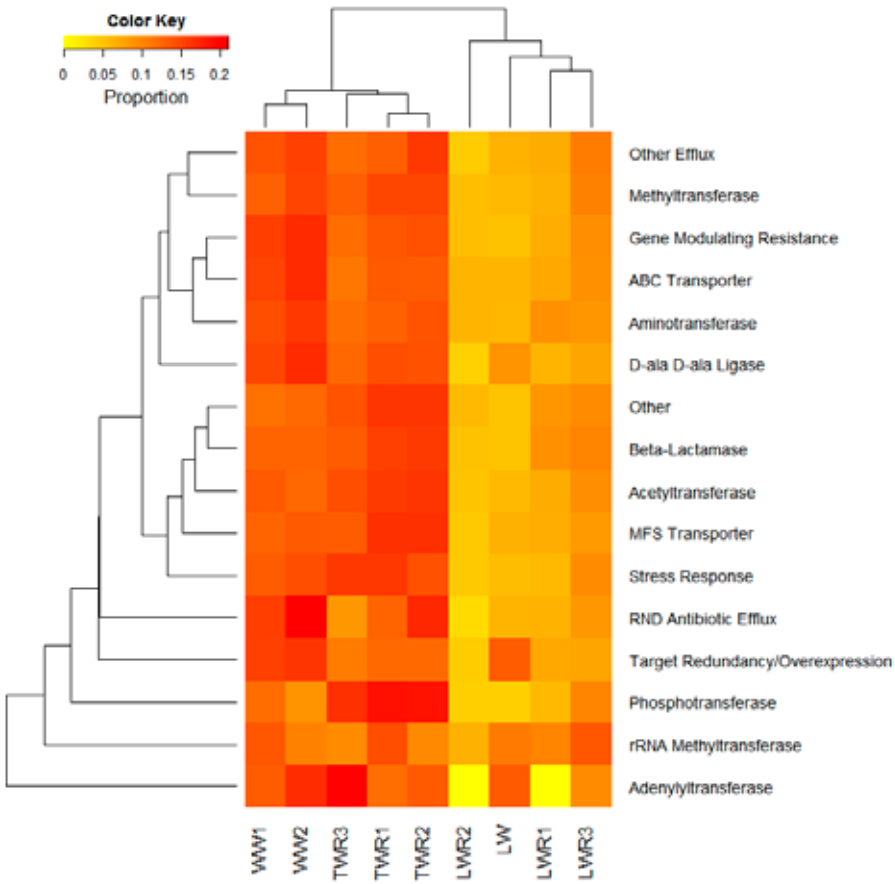

Figure 15. Relative abundance of antibiotic resistance genes present in wastewater (WW1-2), lake water (LW), tap water reactor (TWR1-3) and lake water reactor samples (LWR1-3) by Resfams mechanism in the analyzed metagenomes.

5 Conclusions

Algae were able to grow successfully in a wastewater medium in the various conditions tested, and lake water inoculation had positive effects on the algal growth (Paper I-IV). The impact of addition of lake water to photobioreactors on the performance of the reactors was dependent on the season when the lake water was collected. Sampling the lake water for inoculum during summer resulted in higher and faster algal growth, most likely due to a more active algae starter culture (Paper I).

The reactors were effective in removing ammonium and phosphorous from the wastewater. After 16 days of cultivation, both nitrogen and phosphorous levels in the water phase were below Swedish effluent standards (Papers I and III). While 16 days is too long for a full-scale system to be effective, the treatment time can be reduced by moving to a semi-continuous or a continuous system and by optimizing the growth conditions.

Out of the studied metals, the photobioreactors were successful in reducing the concentrations of Co and Zn while Cr, Ni, Cu, As, Cd and Pb concentrations stayed relatively unchanged or even increased in some cases (Paper III).

Overall, the bacterial populations in the photobioreactors decreased as the concentration of algae increased. This was probably due to competition for the ecological niches in the photobioreactors. By day 8 bacterial 16S rDNA gene copy numbers stabilized and the chlorophyll a concentration had begun to increase (Papers I and III).

Nitrification played an important role in lowering the ammonium concentration. When nitrification was inhibited algal growth rate increased as ammonium was preferred to nitrate as a source of nitrogen for the algae. The rate of uptake of nitrogen and phosphorous was not affected by the inhibition of nitrification although the nitrogen speciation was different, with nitrogen being mostly in the form of ammonium in the inhibited reactors and nitrate in the control (Paper II).

The most dominant algae in the photobioreactors were *Scenedesmus, Desmodesmus* and *Chlorella*, which are commonly seen in wastewater treating photobioreactors and can potentially be used for the production of biogas and biodiesel. The algal community also differed between the reactors inoculated with wastewater and those that did not contain wastewater. The reactors that were not inoculated contained mostly algae belonging to *Chlorella* and *Nitzschia* genera (Papers I and III).

The most abundant bacterial phyla were *Proteobacteria* and *Bacteroidetes*. These results are in line with previous research on algal-bacterial interactions and reinforces our understanding of the most important species in mixed culture photobioreactor communities (Papers III and IV).

The lake water inoculated reactors had more abundant genes for subsystems dealing with photosynthesis, vitamin synthesis and cofactors and fewer controlling virulence and nitrogen metabolism than the tap water reactors and pure wastewater. The bacterial community in the lake water reactors contained more subsystems involved in synthesis of vitamins essential for auxotrophic algae growth. (Papers III and IV).

Nitrification gene abundance was significantly lower in the photobioreactors containing tap water and lake water than in pure wastewater samples. Tap water reactors also had a significantly higher abundance of genes involved in the reduction of nitrate to ammonia (Paper IV).

Pathogens and antibiotic resistance genes were reduced in abundance in the photobioreactors compared to the inflowing wastewater, especially in the reactors inoculated with lake water, however additional analysis is needed to demonstrate statistical significance (Paper IV).

6 Limitations and future work

This thesis only investigated algal and bacterial populations in lab-scale batch reactors, thus the major limitations are related to constraints of the scale. In a large scale continuous flow photobioreactor system the microbial communities may be different as new wastewater is constantly flowing in. However, future studies of full scale systems can benefit from this research since the dominant algae and bacteria were identified during these experiments.

The studies on bacterial and algal interactions in this thesis were performed by analysing the DNA of the organisms, and thus nothing can be said about the expression of the identified genes. Because some DNA from dead and inactive organisms will also be present in the samples, it is also difficult to assess active gene dynamics. This method can identify genes with important or interesting characteristics, however deeper analysis via transcriptomics and proteomics is required to determine if and how they are used by the organisms. Additional biochemical analysis is also required to detect the synthesis of key chemicals such as vitamins.

There are still many steps remaining in the process of understanding the microbiology of photobioreactors, and this thesis presents only a small part of the full story. However, the genetic data that was produced alongside chemical and environmental analyses in this study can be helpful for future studies of full scale systems and to create new tools such as primers for faster analysis.

References

Alcántara, C., Domínguez, J.M., García, D., Blanco, S., Pérez, R., García-Encina, P. a., Muñoz, R., 2015. Evaluation of wastewater treatment in a novel anoxic-aerobic algal-bacterial photobioreactor with biomass recycling through carbon and nitrogen mass balances. Bioresour. Technol. 191, 173–186.

Amin, S. a., Parker, M.S., Armbrust, E. V., 2012. Interactions between Diatoms and Bacteria. Microbiol. Mol. Biol. Rev. 76, 667–684.

Assemany, P.P., Calijuri, M.L., Couto, E.D.A. Do, de Souza, M.H.B., Silva, N.C., Santiago, A.D.F., Castro, J.D.S., 2015. Algae/bacteria consortium in high rate ponds: Influence of solar radiation on the phytoplankton community. Ecol. Eng. 77, 154–162.

Awuah, E., 2006. Pathogen removal mechanisms in macrophyte and algal waste stabilization ponds. Taylor & Francis/Balkema, Leiden, The Netherlands.

Behrens, P.W., 2011. Photobioreactors and Fermentors: The Light and Dark Sides of Growing Algae, in: Andersen, R. (Ed.), Algal Culturing Techniques. Elsevier Academic Press.

Bell, W., Mitchell, R., 1972. Chemotactic and Growth Responses of Marine Bacteria to Algal Extracellular Products. Biol. Bull. 143, 265–277.

Bellinger, E.G., Sigee, D.C., 2010. Freshwater Algae Identification and Use as Bioindicators. John Wiley & Sons, Ltd., Chichester, West Sussex, PO19 8SQ, UK.

Benemann, J., 2013. Microalgae for Biofuels and Animal Feeds. Energies 6 (11), 5869-5886.

Brembu, T., Winge, P., Tooming-Klunderud, A., Nederbragt, A.J., Jakobsen, K.S., Bones, A.M., 2014. The chloroplast genome of the diatom Seminavis robusta: New features introduced through multiple mechanisms of horizontal gene transfer. Mar. Genomics 16, 17–27.

Cabanelas, I.T.D., Arbib, Z., Chinalia, F. a., Souza, C.O., Perales, J. a., Almeida, P.F., Druzian, J.I., Nascimento, I.A., 2013. From waste to energy: Microalgae production in wastewater and glycerol. Appl.

Energy 109, 283–290.
Carney, L.T., Reinsch, S.S., Lane, P.D., Solberg, O.D., Jansen, L.S., Williams, K.P., Trent, J.D., Lane, T.W., 2014. Microbiome analysis of a microalgal mass culture growing in municipal wastewater in a prototype OMEGA photobioreactor. Algal Res. doi:10.1016/j.algal.2013.11.006
Çetinkaya Dönmez, G., Aksu, Z., Öztürk, a, Kutsal, T., 1999. A comparative study on heavy metal biosorption characteristics of some algae. Process Biochem. 34, 885–892.
Chen, C.-Y., Lin, J.-H., 2006. Toxicity assessment of pesticides to Pseudokirchneriella subcapitata under air-tight test environment. J. Hazard. Mater. 131, 6–12.
Cho, S., Lee, N., Park, S., Yu, J., Luong, T.T., Oh, Y.-K., Lee, T., 2013. Microalgae cultivation for bioenergy production using wastewaters from a municipal WWTP as nutritional sources. Bioresour. Technol. 131, 515–520.
Choi, O., Das, A., Yu, C.-P., Hu, Z., 2010. Nitrifying bacterial growth inhibition in the presence of algae and cyanobacteria. Biotechnol. Bioeng. 107, 1004–1011.
Christenson, L., Sims, R., 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnol. Adv. 29, 686–702.
Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G., 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438, 90–93.
de-Bashan, L.E., Hernandez, J.-P., Morey, T., Bashan, Y., 2004. Microalgae growth-promoting bacteria as “helpers” for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater. Water Res. 38, 466–474.
Di Termini, I., Prassone, A., Cattaneo, C., Rovatti, M., 2011. On the nitrogen and phosphorus removal in algal photobioreactors. Ecol. Eng. 37, 976–980.
Dortch, Q., 1990. The interaction between ammonium and nitrate uptake in phytoplankton . Mar. Ecol. Prog. Ser. 61, 183–201.
Escapa, C., Coimbra, R.N., Paniagua, S., García, a. I., Otero, M., 2015. Nutrients and pharmaceuticals removal from wastewater by culture and harvesting of Chlorella sorokiniana. Bioresour. Technol. 185, 276–284.

Ferrero, E.M., de Godos, I., Rodríguez, E.M., García-Encina, P. a., Muñoz, R., Bécares, E., 2012. Molecular characterization of bacterial communities in algal–bacterial photobioreactors treating piggery wastewaters. Ecol. Eng. 40, 121–130.

Gadd, G.M., 2004. Microbial influence on metal mobility and application for bioremediation. Geoderma 122, 109–119.

Gibson, M.K., Forsberg, K.J., Dantas, G., 2014. Improved annotation of antibiotic resistance determinants reveals microbial resistomes cluster by ecology. ISME J. 1–10.

Goecke, F., Labes, A., Wiese, J., Imhoff, J.F., 2010. Chemical interactions between marine macroalgae and bacteria. Mar. Ecol.-Prog. Ser. 409, 267–299.

González, C., Marciniak, J., Villaverde, S., García-Encina, P. a., Muñoz, R., 2008. Microalgae-based processes for the biodegradation of pretreated piggery wastewaters. Appl. Microbiol. Biotechnol. 80, 891–898.

Gonzalez, L.E., Bashan, Y., 2000. Increased growth of the microalga Chlorella vulgaris when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium Azospirillum brasilense. Appl. Environ. Microbiol. 66, 1527–1531.

González-Fernández, C., Riaño-Irazábal, B., Molinuevo-Salces, B., Blanco, S., García-González, M.C., 2011. Effect of operational conditions on the degradation of organic matter and development of microalgae-bacteria consortia when treating swine slurry. Appl. Microbiol. Biotechnol. 90, 1147–1153.

Gurevich, A., Saveliev, V., Vyahhi, N., Tesler, G., 2013. QUAST: Quality assessment tool for genome assemblies. Bioinformatics 29, 1072–1075.

Harms, G., Layton, A.C., Dionisi, H.M., Gregory, I.R., Garrett, V.M., Hawkins, S.A., Robinson, K.G., Sayler, G.S., 2003. Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environ. Sci. Technol. 37, 343–351.

Hauswedell, H., Singer, J., Reinert, K., 2014. Lambda: the local aligner for massive biological data. Bioinformatics 30, 349–355.

Helliwell, K.E., Wheeler, G.L., Smith, A.G., 2015. Widespread decay of vitamin-related pathways: coincidence or consequence? Trends Genet. 29, 469–478.

Henkanatte-Gedera, S.M., Selvaratnam, T., Caskan, N., Nirmalakhandan, N., Van Voorhies, W., Lammers, P.J., 2015. Algal-based, Single-step

Treatment of Urban Wastewaters. Bioresour. Technol. 189, 273–278.
Hughes, D.T., Sperandio, V., 2008. Inter-kingdom signalling: communication between bacteria and their hosts. Nat. Rev. Microbiol. 6, 111–120.
Ju, F., Guo, F., Ye, L., Xia, Y., Zhang, T., 2014. Metagenomic analysis on seasonal microbial variations of activated sludge from a full-scale wastewater treatment plant over 4 years. Environ. Microbiol. Rep. 6, 80–89.
Karya, N.G. a I., van der Steen, N.P., Lens, P.N.L., 2013. Photo-oxygenation to support nitrification in an algal-bacterial consortium treating artificial wastewater. Bioresour. Technol. 134, 244–50.
Kesaano, M., Sims, R.C., 2014. Algal biofilm based technology for wastewater treatment. Algal Res. doi:10.1016/j.algal.2014.02.003
Knud-Hansen, C.F., 1998. Pond Fertilization: Ecological Approach and Practical Application. Oregon State University, Snell Hall, Corvallis, OR, USA.
Komolafe, O., Velasquez Orta, S.B., Monje-Ramirez, I., Noguez, I.Y., Harvey, A.P., Orta Ledesma, M.T., 2013. Biodiesel production from indigenous microalgae grown in wastewater. Bioresour. Technol. 154C, 297–304.
Kouzuma, A., Watanabe, K., 2015. Exploring the potential of algae/bacteria interactions. Curr. Opin. Biotechnol. 33, 125–129.
Krohn-Molt, I., Wemheuer, B., Alawi, M., Poehlein, A., Güllert, S., Schmeisser, C., Pommerening-Röser, A., Grundhoff, A., Daniel, R., Hanelt, D., Streit, W.R., 2013. Metagenome survey of a multispecies and alga-associated biofilm revealed key elements of bacterial-algal interactions in photobioreactors. Appl. Environ. Microbiol. 79, 6196–
Kvarnäs, H., 2001. Morphometry and hydrology of the four large lakes of Sweden. Ambio 30, 467–74.
Lakaniemi, A.-M., Hulatt, C.J., Wakeman, K.D., Thomas, D.N., Puhakka, J. a, 2012. Eukaryotic and prokaryotic microbial communities during microalgal biomass production. Bioresour. Technol. 124, 387–93.
Le Chevanton, M., Garnier, M., Bougaran, G., Schreiber, N., Lukomska, E., Bérard, J.-B., Fouilland, E., Bernard, O., Cadoret, J.-P., 2013. Screening and selection of growth-promoting bacteria for Dunaliella cultures. Algal Res. 2, 212–222.
Li, D., Liu, C.-M., Luo, R., Sadakane, K., Lam, T.-W., 2015. MEGAHIT:

An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics. 31(10), 1674-1676.

Mackenzie, C., Eraso, J.M., Choudhary, M., Roh, J.H., Zeng, X., Bruscella, P., Puskás, Á., Kaplan, S., 2007. Postgenomic Adventures with *Rhodobacter sphaeroides* *. Annu. Rev. Microbiol. 61, 283–307.

Matsuo, Y., Imagawa, H., Nishizawa, M., Shizuri, Y., 2005. Isolation of an Algal Morphogenesis. Science 307, 1598.

Moszczyński, K., MacKiewicz, P., Bodył, A., 2012. Evidence for horizontal gene transfer from bacteroidetes bacteria to dinoflagellate minicircles. Mol. Biol. Evol. 29, 887–892.

Muñoz, R., Guieysse, B., 2006. Algal-bacterial processes for the treatment of hazardous contaminants: a review. Water Res. 40, 2799–2815.

Mussgnug, J.H., Klassen, V., Schlüter, a, Kruse, O., 2010. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. J. Biotechnol. 150, 51–56.

Nõlvak, H., Truu, M., Truu, J., 2012. Evaluation of quantitative real-time PCR workflow modifications on 16S rRNA and tetA gene quantification in environmental samples. Sci. Total Environ. 426, 351–358.

Olguín, E.J., 2012. Dual purpose microalgae-bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a biorefinery. Biotechnol. Adv. 30, 1031–1046.

Overbeek, R., Begley, T., Butler, R.M., Choudhuri, J. V, Chuang, H.-Y., Cohoon, M., de Crécy-Lagard, V., Diaz, N., Disz, T., Edwards, R., Fonstein, M., Frank, E.D., Gerdes, S., Glass, E.M., Goesmann, A., Hanson, A., Iwata-Reuyl, D., Jensen, R., Jamshidi, N., Krause, L., Kubal, M., Larsen, N., Linke, B., McHardy, A.C., Meyer, F., Neuweger, H., Olsen, G., Olson, R., Osterman, A., Portnoy, V., Pusch, G.D., Rodionov, D. a, Rückert, C., Steiner, J., Stevens, R., Thiele, I., Vassieva, O., Ye, Y., Zagnitko, O., Vonstein, V., 2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Res. 33, 5691–5702.

Park, J., Seo, J., Kwon, E.E., 2012. Microalgae Production Using Wastewater: Effect of Light-Emitting Diode Wavelength on Microalgal Growth. Environ. Eng. Sci. 29, 995–1001.

Parks, D.H., Beiko, R.G., 2010. Identifying biologically relevant differences

between metagenomic communities. Bioinformatics 26, 715–721.
Passos, F., Solé, M., García, J., Ferrer, I., 2013. Biogas production from microalgae grown in wastewater: Effect of microwave pretreatment. Appl. Energy 108, 168–175.
Posadas, E., Morales, M.D.M., Gomez, C., Acién, F.G., Muñoz, R., 2015. Influence of pH and CO2 source on the performance of microalgae-based secondary domestic wastewater treatment in outdoors pilot raceways. Chem. Eng. J. 265, 239–248.
Prestat, E., David, M.M., Hultman, J., Ta , N., Lamendella, R., Dvornik, J., Mackelprang, R., Myrold, D.D., Jumpponen, a., Tringe, S.G., Holman, E., Mavromatis, K., Jansson, J.K., 2014. FOAM (Functional Ontology Assignments for Metagenomes): a Hidden Markov Model (HMM) database with environmental focus. Nucleic Acids Res. 42, e145.
Quinn, G.R., Skerman, V.B.D., 1980. Herpetosiphon—Nature's scavenger? Curr. Microbiol. 4, 57–62.
Rawat, I., Ranjith Kumar, R., Mutanda, T., Bux, F., 2013. Biodiesel from microalgae: A critical evaluation from laboratory to large scale production. Appl. Energy 103, 444–467.
Riaño, B., Hernández, D., García-González, M.C., 2012. Microalgal-based systems for wastewater treatment: Effect of applied organic and nutrient loading rate on biomass composition. Ecol. Eng. 49, 112–117.
Romera, E., González, F., Ballester, a, Blázquez, M.L., Muñoz, J. a, 2007. Comparative study of biosorption of heavy metals using different types of algae. Bioresour. Technol. 98, 3344–3353.
Ruiz-Marin, A., Mendoza-Espinosa, L.G., Stephenson, T., 2010. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. Bioresour. Technol. 101, 58–64.
Shi, C.Y., 2011. Mass flow and energy efficiency of municipal wastewater treatment plants. IWA Publishing, London.
Sivakumar, G., Xu, J., Thompson, R.W., Yang, Y., Randol-Smith, P., Weathers, P.J., 2012. Integrated green algal technology for bioremediation and biofuel. Bioresour. Technol. 107, 1–9.
Subashchandrabose, S.R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., Naidu, R., 2011. Consortia of cyanobacteria/microalgae and bacteria: biotechnological potential. Biotechnol. Adv. 29, 896–907.
Suresh Kumar, K., Dahms, H.-U., Lee, J.-S., Kim, H.C., Lee, W.C., Shin,

K.-H., 2014. Algal photosynthetic responses to toxic metals and herbicides assessed by chlorophyll a fluorescence. Ecotoxicol. Environ. Saf. 104C, 51–71.

Zhou, W., Chen, P., Min, M., Ma, X., Wang, J., Griffith, R., Hussain, F., Peng, P., Xie, Q., Li, Y., Shi, J., Meng, J., Ruan, R., 2014. Environment-enhancing algal biofuel production using wastewaters. Renew. Sustain. Energy Rev. 36, 256–269.

Tang, H., Chen, M., Garcia, M.E.D., Abunasser, N., Ng, K.Y.S., Salley, S.O., 2011. Culture of microalgae Chlorella minutissima for biodiesel feedstock production. Biotechnol. Bioeng. 108, 2280–2287.

Travieso, L., Cañizares, R.O., Borja, R., Benítez, F., Domínguez, a R., Dupeyrón, R., Valiente, V., 1999. Heavy metal removal by microalgae. Bull. Environ. Contam. Toxicol. 62, 144–151.

Wang, B., Lan, C.Q., 2011. Biomass production and nitrogen and phosphorus removal by the green alga Neochloris oleoabundans in simulated wastewater and secondary municipal wastewater effluent. Bioresour. Technol. 102, 5639–5644.

Willén, E., 1987. Phytoplankton and reversed eutrophication in Lake Mälaren, central Sweden, 1965–1983. Br. Phycol. J. 37–41.

Wood, D.E., Salzberg, S.L., 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 15, R46.

Wu, Y.-H., Hu, H.-Y., Yu, Y., Zhang, T.-Y., Zhu, S.-F., Zhuang, L.-L., Zhang, X., Lu, Y., 2014. Microalgal species for sustainable biomass/lipid production using wastewater as resource: A review. Renew. Sustain. Energy Rev. 33, 675–688.