Joint PhD degree in Environmental Technology

Docteur de l'Université Paris-Est
Spécialité : Science et Technique de l'Environnement

Dottore di Ricerca in Tecnologie Ambientali

Degree of Doctor in Environmental Technology

Thesis for the degree of Doctor of Philosophy in Environmental Technology

# Tesi di Dottorato – Thèse – PhD thesis – Väitöskirja

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# Nutrient and organic matter removal from wastewaters with microalgae

*22/05/2019, Tampere*

In front of the PhD evaluation committee

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Marie Sklodowska-Curie European Joint Doctorate, Advanced Biological Waste-to-Energy Technologies

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This research was conducted in the framework of the Marie Sklodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-to-Energy Technologies (ABWET) and supported by from Horizon 2020 under grant agreement no. 643071.

# Abstract

Use of microalgae in wastewater treatment has been increasingly studied to integrate with or replace the present treatment systems for removal of nutrients and other pollutants. The potential advantages of this integration (wastewater treatment and microalgal cultivation) could be simultaneous recovery of nitrogen and phosphorus and the use of produced microalgal biomass as feedstock for e.g. biofuel, fertilizer and/or energy. However, the use of microalgae in wastewater treatment is mainly in research stage due to e.g. low nutrient removal and microalgal biomass growth. The aim of this thesis was to enable efficient nutrient and organic matter removal from wastewaters by microalgae while promoting microalgal biomass production.

*Chlorella vulgaris* and *Scenedesmus acuminatus* were successfully grown in batch photobioreactors with liquid digestates from anaerobic digestion (AD) of biosludge from a municipal wastewater treatment plant (ADMW) and a pulp and paper mill wastewater treatment plant (ADPP). The final ammonium removal efficiencies were above 97% when cultivating both microalgae separately in ADPP, however, only 24% and 44% of ammonium were removed from ADMW by *C. vulgaris* and *S. acuminatus*, respectively. Both microalgae efficiently removed phosphate (>96%), while color (74–80%) and soluble COD (27–39%) were partially removed from ADMW and ADPP.

The obtained highest *S. acuminatus* biomass concentration (7.8–10.8 g $L^{-1}$ VSS) in ADPP is among the highest yields reported for microalgae in real wastewaters. Higher *S. acuminatus* biomass yields were obtained in thermophilic ADPP (without and with pretreatment prior to AD: 10.2±2.2 and 10.8±1.2 g $L^{-1}$, respectively) than in pretreated mesophilic ADPP (7.8±0.3 g $L^{-1}$). In addition, the highest microalgal biomass concentration and methane yields were obtained in the same integrated AD and microalgal cultivation system (thermophilic AD with pretreatment).

The iron (0.1, 1.0, and 1.9 mg $L^{-1}$) and sulfate-sulfur (3.7, 20, and 35.8 mg $L^{-1}$) concentrations were found to affect nitrogen removal efficiency and microalgal biomass concentration more in the media with nitrate than with ammonium, probably due to different microalgal assimilation mechanisms for nitrate and ammonium. In this study, synthetic medium with nitrate as nitrogen source with 1.0 mg $L^{-1}$ iron and 35.8 mg $L^{-1}$ sulfate-sulfur enabled the highest microalgal biomass concentration. The effect of iron concentration on nitrate removal efficiency and microalgal growth was more significant than that of

sulfate concentration, while the interaction effect between sulfate and iron was not observed.

The average ammonium removal efficiency (14 to 30%) and microalgal biomass concentration (0.50 to 1.17 g particulate organic carbon per L) in continuous-flow membrane photobioreactor were promoted by adding a low concentration of zeolite (0.5 g $L^{-1}$). The zeolite likely provided a habitat for attached growth of microalgae and high availability of ammonium for growth on the surface of the zeolite due to ammonium adsorption to zeolite. Further increase in zeolite concentration (from 0.5 to 1 and 5 g $L^{-1}$) did not improve ammonium removal efficiency or biomass concentration. This was likely due to the increased solution turbidity caused by breaking apart of added zeolite particles into finer particles, which reduced light availability.

In summary, this work showed the possibility of utilizing microalgae in wastewater treatment to efficiently remove nutrients and organic matter, and simultaneously promote microalgal growth. Selecting suitable microalgal species for the specific wastewater to remove nutrients and organic matter is essential to promote algae-based wastewater treatment applications.

# Tiivistelmä

Mikroleviä voidaan hyödyntää jätevesien käsittelyssä nykyisten käsittelyjärjestelmien yhteydessä tai kokonaan korvaamaan nykyiset käsittelymenetelmät ravinteiden ja muiden epäpuhtauksien poistossa. Jätevedenkäsittelyn ja mikrolevien kasvatuksen yhdistäminen mahdollistaa typen ja fosforin talteenoton ja samanaikaisesti tuotetun mikroleväbiomassan hyödyntämisen esimerkiksi biopolttoaineiden ja/tai lannoitteiden raaka-aineena. Mikrolevien käyttö jätevedenkäsittelyssä vaatii kuitenkin vielä tutkimus- ja kehitystyötä ravinteiden poistotehokkuuden ja mikrolevien kasvun tehostamiseksi. Tämän väitöskirjan tavoitteena oli mahdollistaa tehokas ravinteiden ja orgaanisen aineksen poisto jätevesistä edistäen samalla mikrolevien tehokasta kasvua.

Laboratoriomittakaavan fotobioreaktoreissa tehdyissä panoskokeissa *Chlorella vulgaris* ja *Scenedesmus acuminatus* mikrolevien todettiin kasvavan sekä kunnallisen (ADMW) että sellu- ja paperitehtaan (ADPP) jätevedenpuhdistamon ylijäämälietteen mädätyksen rejektivesissä. Kummankin levän avulla pystyttiin poistamaan yli 97% ADPP:n sisältämästä ammoniumista, mutta ADMW:sta ammoniumpoistotehokkuus oli vain 24 % kasvatettaessa *C. vulgaris* mikrolevää ja 44 % kasvatettaessa *S. acuminatus* mikrolevää. Molempien mikrolevien fosforinpoistotehokkuus kummastakin rejektivedestä oli yli 96 %. Myös väriä (74-80 %) ja kemiallinen hapenkulutusta (27-39 %) saatiin vähennettyä.

Kokeissa ADPP:ssa saavutetut *S. acuminatus* biomassakonsentraatiot (7,8-10,8 g $L^{-1}$ VSS) ovat korkeimpien joukossa, kun vertaillaan oikeita jätevesiä käytettäessä kirjallisuudessa raportoituja mikroleväbiomassasaantoja. Vertailtaessa *S. acuminatus* mikrolevän kasvua eri mädätysolosuhteissa tuotetuissa ADPP rejektivesissä, suurin *S. acuminatus* biomassakonsentraatio saavutettiin termofiilisen mädätyksen rejektivesissä. Ilman esikäsittelyä ennen termofiilistä mädätystä korkein biomassakonsentraatio oli 10,2 ± 2,2 g $L^{-1}$ ja esikäsittelyn sisältäneen termofiilisen mädätyksen rejektivedessä 10,8 ± 1,2 g $L^{-1}$. Esikäsittelyn sisältäneen mesofiilisen mädätyksen ADPP-rejektivedessä suurin *S. acuminatus* biomassakonsentraatio oli 7,8 ± 0,3 g $L^{-1}$. Myös korkein metaanin tuotto saavutettiin esikäsittelyn sisältäneessä termofiilisessä mädätysprosessissa, mikä osoittaa, että tehokkain metaanin tuotto ja mikrolevien biomassatuotto saavutettiin samoissa prosessiolosuhteissa.

Raudan (0,1; 1,0 ja 1,9 mg $L^{-1}$) ja sulfaatti- rikin (3;7; 20 ja 35,8 mg $L^{-1}$) pitoisuuksien havaittiin vaikuttavan typen poistotehokkuuteen ja mikrolevien biomassakonsentraatioon enemmän typenlähteen ollessa nitraatti kuin käytettäessä ammoniumia typenlähteenä.

Korkein *S. acuminatus* biomassakonsentraatio saavutettiin nitraattipohjaisessa kasvatusmediassa, jossa oli 1,0 mg $L^{-1}$-rautaa ja 35,8 mg $L^{-1}$ rikkiä. Rautakonsentraatio vaikutti mikrolevien kasvuun ja typenpoistotehokkuuteen enemmän kuin sulfaattipitoisuus. Raudalla ja rikillä ei havaittu olevan yhteisvaikutusta.

Mikrolevien kasvua pyrittiin tehostamaan lisäämällä jatkuvatoimiseen membraanifotobioreaktoriin eri määriä zeoliiittia. Kun zeoliittia lisättiin 0,5 g $L^{-1}$, keskimääräinen ammoniumin poistotehokkuus nousi 14 %:sta 30 %:iin ja biomassakonsentraatio 0.5 g $L^{-1}$:sta yli 1,0 g $L^{-1}$:aan. Havaitun tehokkuuden lisääntymisen uskottiin johtuvan siitä, että zeoliitti tarjosi pinnan, jolla mikrolevien havaittiin kasvavan. Lisäksi zeoliitin on osoitettu adsorboivan ammoniumia ympäröivästä vedestä. Reaktorin zeoliittikonsentraation nostaminen 0.5 g $L^{-1}$:sta 1g $L^{-1}$:aan ja myöhemmin 5 g $L^{-1}$:aan ei kuitenkaan enää kasvattanut ammoniumin poistotehokkuutta tai biomassakonsentraatiota. Tämä johtui todennäköisesti zeoliittipartikkelien hajoamisesta hienommiksi hiukkasiksi, mikä heikensi valon saatavuutta.

Tutkimus osoitti, että mikroleviä voidaan hyödyntää jätevedenpuhdistuksessa ravinteiden talteenottoon ja samalla kasvattaa tehokkaasti mikroleväbiomassaa. On kuitenkin tärkeää valita kullekin jätevedelle soveltuva mikrolevälaji, jotta prosessi toimisi tehokkaasti.

# Résumé

L'utilisation de micro-algues pour le traitement des eaux usées est de plus en plus étudiée pour intégrer ou remplacer les systèmes de traitement actuel permettant d'éliminer les nutriments et autres polluants carbonés. Les avantages potentiels de cette intégration (traitement des eaux usées et culture de micro-algues) pourraient être une récupération simultanée de l'azote et du phosphore et l'utilisation de la biomasse de micro-algues produite comme matière première pour la production, par exemple, de biocarburant, d'engrais et / ou d'énergie. Cependant, l'utilisation des micro-algues pour le traitement des eaux usées est toujours au stade de la recherche à cause de faibles rendements d'élimination des nutriments et faibles taux de croissance de la biomasse des micro-algues. Le but de cette thèse était de permettre l'élimination efficace des éléments nutritifs et de la matière organique des eaux usées par les micro-algues tout en favorisant la production de biomasse de micro-algues.

*Chlorella vulgaris* et *Scenedesmus acuminatus* ont été cultivés avec succès dans des photobioréacteurs discontinus contenant des digestats liquides issus de la digestion anaérobie (AD) de boues biologiques provenant d'une station d'épuration municipale (ADMW) et d'une usine de traitement des eaux usées d'une papeterie (ADPP). Les rendements finaux d'élimination de l'ammonium sont supérieurs à 97% lorsque les deux micro-algues sont cultivées séparément dans le digestat de l'ADPP. Toutefois, seuls 24% et 44% de l'ammonium ont été éliminés du digestat de l'ADMW par *C. vulgaris* et *S. acuminatus*, respectivement. Les deux micro-algues ont efficacement éliminé le phosphate (> 96%), tandis que la couleur (74–80%) et la DCO soluble (27–39%) ont été partiellement éliminées des digestats de l'ADMW et de l'ADPP.

La concentration de biomasse la plus élevée obtenue pour *S. acuminatus* (7,8 à 10,8 g $L^{-1}$ MVS) dans l'ADPP figure parmi les valeurs les plus élevées et rapportées pour les micro-algues dans les eaux usées réelles. Des concentrations supérieures en biomasse de *S. acuminatus* ont été obtenus pour les digestats d'ADPP obtenus en condition thermophile (sans et avec prétraitement avant digestion anaérobie: 10,2 ± 2,2 et 10,8 ± 1,2 g $L^{-1}$, respectivement) par rapport aux digestats d'ADPP obtenus en condition mésophile prétraité (7,8 ± 0,3 g $L^{-1}$). De plus, les concentrations les plus élevées en biomasse de micro-algues et en méthane ont été obtenues dans le même système intégré de digestion anaérobie et de culture de micro-algues (AD thermophile avec prétraitement).

Les concentrations en fer (0,1, 1,0 et 1,9 mg $L^{-1}$) et sulfate (3,7, 20 et 35,8 mg $L^{-1}$) affectent davantage l'efficacité de l'élimination de l'azote et la concentration de la biomasse de micro-algues dans les milieux en présence de nitrates qu'avec l'ammonium, probablement en raison de différents mécanismes d'assimilation des micro-algues pour les nitrates et l'ammonium. Dans cette étude, un milieu synthétique contenant du nitrate comme source d'azote avec 1,0 mg de $L^{-1}$ de fer et 35,8 mg de $L^{-1}$ de sulfate permet d'obtenir la plus forte concentration de biomasse de micro-algues. L'effet de la concentration de fer sur l'efficacité d'élimination des nitrates et la croissance des micro-algues était plus important que celui de la concentration en sulfate, alors que l'effet d'interaction entre le sulfate et le fer n'a pas été observé.

L'efficacité moyenne d'élimination de l'ammonium (14 à 30%) et la concentration de biomasse de micro-algues (0,50 à 1,17 g de carbone organique particulaire par litre) dans le photobioréacteur à flux continu ont été améliorées par l'ajout d'une faible concentration de zéolite (0,5 g $L^{-1}$). L'ajout de zéolite favorise probablement la croissance de micro-algues en surface du matériau associé à une grande disponibilité d'ammonium pour la croissance à la surface de la zéolite. Une augmentation supplémentaire de la concentration en zéolite (de 0,5 à 1 et 5 g $L^{-1}$) n'a pas amélioré l'efficacité de l'élimination de l'ammonium ni la concentration de la biomasse. Cela est probablement dû à la turbidité accrue de la solution provoquée par la fragmentation des particules de zéolite ajoutées en particules plus fines, ce qui a réduit la pénétration de la lumière dans le photobioréacteur.

En résumé, ces travaux ont montré la possibilité d'utiliser des micro-algues pour le traitement des eaux usées afin d'éliminer efficacement les nutriments et les matières organiques, tout en favorisant la croissance des micro-algues. La sélection d'espèces de micro-algues adaptées aux eaux usées spécifiques pour éliminer les nutriments et les matières organiques est essentielle pour promouvoir les applications de traitement des eaux usées à base de micro-algues.

# Samenvatting

Het gebruik van microalgen in afvalwaterzuivering is in toenemende mate bestudeerd om ingepast te worden in of de huidige behandelingssystemen te vervangen voor het verwijderen van voedingsstoffen en andere verontreinigende stoffen. De potentiële voordelen van deze integratie (afvalwaterzuivering en microalgenkweek) kunnen gelijktijdige terugwinning van stikstof en fosfor zijn en het gebruik van geproduceerde microalgische biomassa als grondstof voor b.v. biobrandstof, kunstmest en / of energie. Het gebruik van microalgen in de behandeling van afvalwater is echter voornamelijk in de onderzoeksfase als gevolg van b.v. lage voedingsstoffenverwijdering en groei van microalgen uit biomassa. Het doel van dit proefschrift is om efficiënte verwijdering van voedingsstoffen en organische stoffen uit afvalwater door microalgen mogelijk te maken en tegelijkertijd de productie van microalgen te bevorderen.

*Chlorella vulgaris* en *Scenedesmus acuminatus* werden met succes gekweekt in batchfotobioreactoren met vloeibare digestaten uit anaërobe digestie (AD) van bioslib van een gemeentelijke afvalwaterzuiveringsinstallatie (ADMW) en een afvalwaterbehandelingsinstallatie voor pulp en papierfabrieken (ADPP). De uiteindelijke ammonium verwijderingsrendementen waren hoger dan 97% wanneer beide microalgen afzonderlijk in ADPP werden gekweekt, maar slechts 24% en 44% ammonium werden verwijderd uit ADMW door respectievelijk *C. vulgaris* en *S. acuminatus*. Beide microalgen verwijderden fosfaat efficiënt (> 96%), terwijl kleur (74-80%) en solitaire COD (27-39%) gedeeltelijk werden verwijderd uit ADMW en ADPP.

De verkregen hoogste biomassaconcentratie van *S. acuminatus* (7,8–10,8 g $L^{-1}$ VSS) in ADPP is een van de hoogste gerapporteerde opbrengsten voor microalgen in echt afvalwater. Hogere opbrengst aan *S. acuminatus* biomassa werd verkregen in thermofiele ADPP (zonder en met voorbehandeling voor AD: 10,2 ± 2,2 en 10,8 ± 1,2 g $L^{-1}$, respectievelijk) dan in voorbehandelde mesofiele ADPP (7,8 ± 0,3 g $L^{-1}$). Bovendien werden de hoogste microalgal biomassaconcentratie en methaanopbrengsten verkregen in hetzelfde geïntegreerde AD en microalgen kweeksysteem (thermofiele AD met voorbehandeling).

De concentraties van ijzer (0,1, 1,0 en 1,9 mg $L^{-1}$) en sulfaat-zwavel (3,7, 20 en 35,8 mg $L^{-1}$) bleken de stikstofverwijderingsefficiëntie en microalgenconcentratie van biomassa meer in de media te beïnvloeden. meer nitraat dan met ammonium, waarschijnlijk als

gevolg van verschillende microalgen assimilatie mechanismen voor nitraat en ammonium. In deze studie maakte synthetisch medium met nitraat als stikstofbron met 1,0 mg $L^{-1}$ ijzer en 35,8 mg $L^{-1}$ sulfaat-zwavel de grootste biomassa-concentratie van microalgen mogelijk. Het effect van ijzerconcentratie op de nitraatverwijderingsefficiëntie en de groei van microalgen was significanter dan dat van de sulfaatconcentratie, terwijl het interactieve effect tussen sulfaat en ijzer niet werd waargenomen.

De gemiddelde ammoniumverwijderingsefficiëntie (14 tot 30%) en microalgen biomassa concentratie (0,50 tot 1,17 g deeltjesvormige organische koolstof per L) in een continu stromende membraan fotobioreactor werden bevorderd door toevoeging van een lage concentratie zeoliet (0,5 g $L^{-1}$). Het zeoliet verschafte waarschijnlijk een levensomgeving ter bevordering van de groei van microalgen en hoge beschikbaarheid van ammonium voor groei op het oppervlak van het zeoliet als gevolg van ammoniumadsorptie aan zeoliet. Verdere toename in zeolietconcentratie (van 0,5 tot 1 en 5 g $L^{-1}$) verbeterde de ammoniumverwijderingsefficiëntie of biomassaconcentratie niet. Dit was hetzelfde vanwege de toegenomen turbiditeit van de oplossing, veroorzaakt door het uiteenvallen van toegevoegde zeolietparels in fijnere deeltjes, wat de beschikbaarheid van licht verminderde.

Samenvattend toonde dit werk de mogelijkheid om microalgen in de afvalwaterzuivering te gebruiken om op een efficiënte manier voedingsstoffen en organisch materiaal te verwijderen en tegelijkertijd de groei van microalgen te stimuleren. Het selecteren van geschikte microalgen soorten voor het specifieke afvalwater om nutriënten en organisch materiaal te verwijderen, is van essentieel belang om op algen gebaseerde toepassingen voor de behandeling van afvalwater te bevorderen.

# Sommario

L'uso di microalghe nel trattamento delle acque reflue è stato sempre più studiato per integrare o sostituire gli attuali sistemi di trattamento per la rimozione di nutrienti e altri inquinanti. I potenziali vantaggi di questa integrazione (trattamento delle acque reflue e coltivazione microalgale) potrebbero essere il recupero simultaneo di azoto e fosforo e l'uso di biomassa prodotta come materia prima per es. biocarburante, fertilizzante e / o energia. Tuttavia, l'uso di microalghe nel trattamento delle acque reflue è principalmente in fase di ricerca a causa, ad es. del basso tasso di rimozione di nutrienti e di crescita della biomassa algale. Lo scopo di questa tesi era quello di consentire un'efficace rimozione dei nutrienti e della sostanza organica dalle acque reflue da parte delle microalghe, promuovendo al tempo stesso la produzione di biomassa algale.

La *Chlorella vulgaris* e lo *Scenedesmus acuminatus* sono stati coltivati con successo in fotobioreattori in batch con digestati liquidi dalla digestione anaerobica (AD) di fanghi biologici da un impianto di trattamento delle acque reflue municipali (ADMW) e da un impianto di trattamento delle acque di scarico di impianti di produzione di polpa di cellulosa e cartiere (ADPP). L'efficienza finale di rimozione dell'ammonio era superiore al 97% quando entrambe le microalghe venivano coltivate separatamente nell'ADPP, tuttavia solo il 24% e il 44% di ammonio è stato rimosso dall'ADMW rispettivamente da *C. vulgaris* e *S. acuminatus*. Entrambe le microalghe hanno efficientemente rimosso il fosfato (> 96%), mentre il colore (74-80%) e il COD solubile (27-39%) sono stati parzialmente rimossi da ADMW e ADPP.

La più alta concentrazione di biomassa di *S. acuminatus* ottenuta (7,8-10,8 g di $L^{-1}$ VSS) nell'ADPP è tra i maggiori rendimenti segnalati per le microalghe nelle acque reflue reali. Rese di biomassa di *S. acuminatus* più elevate sono state ottenute in ADPP termofila (senza e con pretrattamento prima di AD: 10,2 ± 2,2 e 10,8 ± 1,2 g $L^{-1}$, rispettivamente) che in ADPP mesofila pretrattata (7,8 ± 0,3 g $L^{-1}$). Inoltre, le più elevate concentrationi di biomassa e rese di metano sono state ottenute nello stesso sistema integrato AD ed il sistema di coltura di microalghe (AD termofilo con pretrattamento).

E' stato riscontrato che le concentrazioni di ferro (0,1, 1,0 e 1,9 mg $L^{-1}$) e solfato-zolfo (3,7, 20 e 35,8 mg $L^{-1}$) influenano l'efficienza di rimozione dell'azoto e la concentrazione di biomassa algale maggiormente nei terreni di coltura con nitrato che con ammonio, probabilmente a causa di diversi meccanismi di assimilazione di nitrati e ammonio da parte delle microalghe. In questo studio, il terreno sintetico con nitrato come fonte di

azoto con 1,0 mg di ferro $L^{-1}$ e 35,8 mg di solfato di sodio $L^{-1}$ ha permesso di raggiungere la più alta concentrazione di biomassa algale. L'effetto della concentrazione del ferro sull'efficienza della rimozione del nitrato e sulla crescita microalgale è stato più significativo di quello della concentrazione di solfato, mentre non è stato osservato l'effetto di interazione tra solfato e ferro.

L'efficienza media di rimozione dell'ammonio (dal 14 al 30%) e la concentrazione di biomassa microalgale (da 0,5 a 1,17 g di carbonio organico particolato per L) nel fotobioreattore a membrana a flusso continuo sono state promosse aggiungendo una bassa concentrazione di zeolite (0,5 g $L^{-1}$). La zeolite probabilmente ha fornito un habitat per la crescita aggregata di microalghe e un'elevata disponibilità di ammonio per la crescita sulla superficie della zeolite dovuta all'adsorbimento dell'ammonio alla zeolite. Un ulteriore aumento della concentrazione di zeolite (da 0,5 a 1 e 5 g $L^{-1}$) non ha migliorato l'efficienza di rimozione dell'ammonio o la concentrazione di biomassa. Ciò è verosimilmente dovuto alla maggiore torbidità della soluzione causata dalla rottura di particelle di zeolite aggiunte in particelle più fini, che hanno ridotto la disponibilità di luce.

In sintesi, questo lavoro ha mostrato la possibilità di utilizzare le microalghe nel trattamento delle acque reflue per rimuovere in modo efficace i nutrienti e la materia organica e contemporaneamente stimolare la crescita microalgale. La selezione di specie microalgali idonee per le specifiche acque reflue per rimuovere sostanze nutritive e materia organica è essenziale per promuovere applicazioni di trattamento delle acque reflue a base di alghe.

# Acknowledgements

The experimental work for this thesis was carried out at Tampere University, Finland; University of South Florida, the USA and IHE Delft, the Netherlands. This work was supported by the Marie Skłodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-To-Energy Technologies (ABWET) funded from European Union Horizon 2020 (grant number 643071). I wish to thank for the funding enabling this thesis.

I would like to thank my supervisor, Prof. Jukka Rintala, for his guidance through my research and for his valuable comments on my work. I gratefully thank my instructor, Asst. Prof. Aino-Maija Lakaniemi, who always supported me and gave me useful comments on my work. I am thankful to my co-supervisors Prof. Eric van Hullebusch and Assoc. Prof. Daniel H. Yeh, and to Dr. Robert Bair, who supervised my research during my exchange periods.

I wish to thank all the past and present co-workers, my colleagues from ABWET European joint degree programme and the ‘membrane’ research group in Tampa. I thank them for the time spent together both during work and free time. I would also thank the technicians, especially Tarja Ylijoki-Kaiste and Antti Nuottajärvi from Tampere University and Peter Heerings from IHE Delft. Finally, I would thank my parents and my partner Savin Gautam for always supporting me.

# Contents

# List of Publications

I. Tao, R., Kinnunen, V., Praveenkumar, R., Lakaniemi, A.M., Rintala, J.A., 2017. Comparison of *Scenedesmus acuminatus* and *Chlorella vulgaris* cultivation in liquid digestates from anaerobic digestion of pulp and paper industry and municipal wastewater treatment sludge. Journal of Applied Phycology, 29(6), 2845-2856.

II. Tao, R., Lakaniemi, A.M., Rintala, J.A., 2017. Cultivation of *Scenedesmus acuminatus* in different liquid digestates from anaerobic digestion of pulp and paper industry biosludge. Bioresource Technology, 245, 706-713.

III. Tao, R., Bair, R., Lakaniemi, A.M., van Hullebusch, E.D., Rintala, J.A., 2019. Use of $2^2$ factorial experimental design to study the effects of iron and sulfate on growth of *Scenedesmus acuminatus* with different nitrogen sources. *Submitted for publication*.

IV. Tao, R., Bair, R., Pickett M., Calabria J., Lakaniemi, A.M., van Hullebusch, E.D., Rintala, J.A., Yeh, D.H., 2019. Low concentration of zeolite to enhance microalgal growth and ammonium removal efficiency in a membrane photobioreactor. *Submitted for publication*.

# Author's Contribution

Paper I: Ran Tao was involved in the design of the study, and carried out the experimental work, all related analyses, data interpretation, and drafting and completion of the manuscript. Viljami Kinnunen and Ramasamy Praveenkumar were involved in the design of the study, the experimental work, and reviewed the manuscript. Aino-Maija Lakaniemi and Jukka A. Rintala were involved in the design of the study, the experimental work, data interpretation, and drafting and completion of the manuscript.

Paper II: Ran Tao was involved in the design of the study, and carried out the experimental work, all related analyses, data interpretation, and drafting and completion of the manuscript. Aino-Maija Lakaniemi and Jukka A. Rintala were involved in the design of the study, the experimental work, data interpretation, and drafting and completion of the manuscript.

Paper III: Ran Tao was involved in the design of the study, and carried out the experimental work, all related analyses, data interpretation, and drafting and completion of the manuscript. Robert Bair and Eric D. van Hullebusch were involved in the design of the study and reviewed the manuscript. Aino-Maija Lakaniemi and Jukka A. Rintala were involved in the design of the study, the experimental work, data interpretation, and drafting and completion of the manuscript.

Paper IV: Ran Tao was involved in the design of the study, and carried out the experimental work, all related analyses, data interpretation, and drafting and completion of the manuscript. Aino-Maija Lakaniemi was involved in the design of the study, the experimental work, data interpretation, and drafting and completion of the manuscript. Eric D. van Hullebusch was involved in the design of the study and data interpretation, and reviewed the manuscript. Jukka A. Rintala was involved in the data interpretation and drafting and completion of the manuscript. Robert Bair, Melanie Pickett, Jorge Calabria and Daniel H. Yeh were involved in the design of the study, experimental work, and data interpretation, and reviewed the manuscript.

# List of Symbols and Abbreviations

| | |
|---|---|
| AD | Anaerobic digestion |
| ADMW | Liquid digestate of municipal wastewater treatment plant biosludge |
| ADPP | Liquid digestate of pulp and paper industry biosludge |
| DOC | Dissolved organic carbon |
| HRT | Hydraulic retention time |
| M | Mesophilic digestate |
| MC | Microalgal cultivation |
| Mp | Pre-treated mesophilic degestate |
| MPBR | Membrane photobioreactor |
| OD | Optical density |
| $OD_{680}$ | Optical density of digestate |
| $OD_{680}$ | Optical density of microalgal biomass |
| POC | Particulate organic carbon |
| SEM-EDX | Scanning electron microscope with Energy Dispersive X-Ray Analysis |
| Soluble $BOD_7$ | Soluble biochemical oxygen demand |
| Soluble COD | Soluble chemical oxygen demand |
| SRT | Solid retention time |
| T | Thermophilic digestate |
| Tp | Pre-treated thermophilic digestate |
| TIC | Total inorganic carbon |
| TOC | Total organic carbon |
| VS | Volatile solids |
| VSS | Volatile suspended solids |

# 1 General Introduction and Thesis Outline

## 1.1 Introduction

Increasing water use, urbanization and population growth have resulted in large amounts of wastewaters, which contain e.g. suspended solids, biodegradable organics, nutrients, metals, and pathogens (Tchobanoglous et al., 2014; Van Drecht et al., 2009). The compositions of wastewaters can vary largely depending on the source (Tchobanoglous et al., 2014). Based on the activities that have resulted in generation of the wastewater, wastewaters can mainly be divided into municipal, industrial, and agricultural wastewaters. Wastewaters typically need treatment prior to being discharged to nature (Tchobanoglous et al., 2014). Organic compounds as well as heavy metals and pathogens can cause negative effects on the environment as well as human and animal health (Melvin and Leusch, 2016; Ratola et al.,2012; Tchobanoglous et al., 2014). Nitrogen and phosphorus are generally present in most wastewaters and they are main contributors to eutrophication as they are essential elements for growth of photosynthetic organisms (Anderson et al., 2002).

A typical wastewater treatment process includes physical, chemical and biological unit processes such as screening, grid removal, sedimentation, activated sludge, and disinfection (Tchobanoglous et al., 2014). The activated-sludge process is an aerobic process, in which aeration is used to supply oxygen for microbes that remove biodegradable organic matter and nutrients from the wastewater. High amount of air is needed for efficient treatment and aeration typically consumes a lot of energy (Tchobanoglous et al., 2014). A traditional way to remove nitrogen from wastewaters is nitrification-denitrification process, where ammonium is oxidized to nitrate and nitrite and then converted into gaseous nitrogen, which is released to the atmosphere (Beuckels et al., 2015; Tchobanoglous et al., 2014). Phosphorus is removed either biologically or by chemical precipitation with iron, alum, or lime

(De-Bashan and Bashan, 2004). In addition, auto-precipitation of phosphorus (like struvite) can occur under certain conditions (such as high pH) depending on the composition of the wastewater (De-Bashan and Bashan, 2004). The sludge generated during the wastewater treatment process typically contains high amounts of nutrients. The typical process of sludge treatment includes e.g. thickening, stabilization, conditioning, dewatering, and transportation (Tchobanoglous et al., 2014). For example, anaerobic digestion is often used for sludge stabilization as produced biogas can be used as bioenergy (Kacprzak et al., 2017). The liquid residues from the sludge treatment (e.g. reject waters form dewatering of digestate) typically contains majority of the nutrients that were present in the wastewater sludge. Traditional treatments such as biological activated-sludge and nitrification–denitrification processes or chemical precipitation process are energy-intensive or resource-intensive (Beuckels et al., 2015; De-Bashan and Bashan, 2004). In addition to aerobic processes, anaerobic processes are increasingly used to treat concentrated industrial wastewaters as well as municipal wastewaters e.g. in India and Brazil to produce biogas, while nutrient removal capacity of anaerobic processes is relatively low (for a review, see Chernicharo et al., 2015).

The traditional wastewater treatment processes have been developed to release water in as clean as possible to the receiving water bodies. However, resource and energy recovery from the wastewaters can contribute to the development of a sustainable society. Nutrient recovery from wastewaters is increasingly considered due to increasing fertiliser consumption caused by population growth and increasing food consumption (for reviews, see Mehta et al., 2015; Kumar et al., 2015). Phosphorus resources in non-renewable phosphate rocks are diminishing, which highligths the importance to recover phosphorus from waste and side streams, as phosphorus cannot be substituted in food production (Cordell et al., 2009). On the other hand, Haber-Bosch process, which is commonly used to produce nitrogen fertilizers, consumes huge amount of energy often produced from fossil sources and therefore generates high greenhouse gas emissions (for a review, see Tanabe and Nishibayashi, 2013).

In recent years, use of microalgae in wastewater treatment has been studied and developed to obtain more sustainable wastewater treatment systems with lower aeration requirement, recovery of nitrogen and phosphorus in utilizable form, and the generation of microalgal biomass that can be used as a feedstock for e.g. biofuel (Sun et al., 2019) and fertilizer production (Coppens et al., 2016). Microalgae are microscopic microorganisms that can carry out photosynthetic activities (Richmond, 2004), and have faster growth rate and use less land areas than terrestrial plants (Clarens et al., 2010). In addition to nutrient recovery, microalgae can remove other pollutants such as organic matter (Di Caprio et al., 2018) and heavy metals (Mane and Bhosle, 2012) from the wastewaters. Using microalgae in wastewater treatment is a promising method to integrate with or replace the present

treatment systems for nutrient removal (Abinandan and Shanthakumar, 2015; Beuckels et al., 2015; De-Bashan and Bashan, 2004).

Use of microalgae in wastewater treatment has been studied using various different wastewaters including municipal, industrial and argicultural wastewaters (for a review, see Cai et al., 2013). However, some problems including low treatment efficiency and high operation costs due to e.g. harvesting have hindered the practical application (for reviews, see Cai et al., 2013; Xia and Murphy, 2016). The pollutant removal efficiency by microalgae can be species-specific, thus, many studies have been carried out to select the suitable species for specific wastewaters (Bohutskyi et al., 2015; Chong et al., 2000). *Chlorella vulgaris* and *Scenedesmus acuminatus* have been studied in municipal and agricultural wastewaters due to their high growth rate and yields, however, their use in studies focusing on industrial wastewater treatment has been rare (Wang et al., 2015; Zuliani et al., 2016). In addition, pretreatments such as sterilization and dilution of the wastewaters are commonly used in laboratory studies due to e.g. bacterial contamination, and high ammonium concentration and turbidity. Further research is needed to solve these challenges to promote the commercialization of microalgal use in wastewater treatment.

## 1.2 Objectives and scope of the study

The objective of the present thesis was to evaluate the feasibility of microalgal monocultures/mixed cultures to remove nutrients and organic matters from wastewaters (liquid digestates). The specific objectives were:

- To assess the feasibility of cultivating *C. vulgaris* and *S. acuminatus* for nutrient removal in liquid digestates from digestion of biosludge originating from a municipal wastewater treatment plant and a pulp and paper mill wastewater treatment plant (Chapter 3 and 4).
- To investigate the effects of different digestion conditions of pulp and paper mill biosludge on nutrient and organic matter removal efficiency from the resulting liquid digestates with *S. acuminatus* (Chapter 4).
- To assess the combined effects of various iron and sulfate concentrations and nitrogen on the ammonium and nitrate removal efficiency and growth of *S. acuminatus* (Chapter 5).
- To assess the effects of adding zeolite at different concentrations on the nutrient removal and growth of mixed microalgal culture in a continuous-flow membrane photobioreactor (Chapter 6).

## 1.3 Thesis outline

This PhD thesis is divided into seven chapters, the main topics of which are shown in Figure 1.1. The first chapter (Chapter 1) provides general background and a brief overview of the thesis. Chapter 2 reviews the current knowledge on microalgae and wastewater treatment, including characteristics of microalgae, microalgal growth requirements, microalgal cultivation systems, characteristics of typical wastewaters, wastewater treatment methods, and findings of recent studies of microalgae use in wastewater treatment. Chapter 3 focuses on the nutrient and organic compound removal by two microalgae *C. vulgaris* and *S. acuminatus* from liquid digestates of two origins – a municipal wastewater treatment plant and a pulp and paper mill wastewater treatment plant. In Chapter 4, the differences of nutrient and organic compound removal by *S. acuminatus* are studied in various types of liquid digestates obtained at different digestion conditions from a pulp and paper mill wastewater treatment plant. Chapter 5 focuses on the combined effects of trace elements (iron and sulfate) on ammonium and nitrate removal efficiency and microalgal growth by using factorial experimental design. In Chapter 6, nutrient removal and microalgal growth are studied in a membrane photobioreactor by adding different concentrations of natural zeolite. The potential benefits and drawbacks of zeolite use in the microalgal cultivations are also discussed. Chapter 7 provides general discussion and conclusions based on the specific research objectives of this thesis and includes future recommendations for the use of microalgae in wastewater treatment.

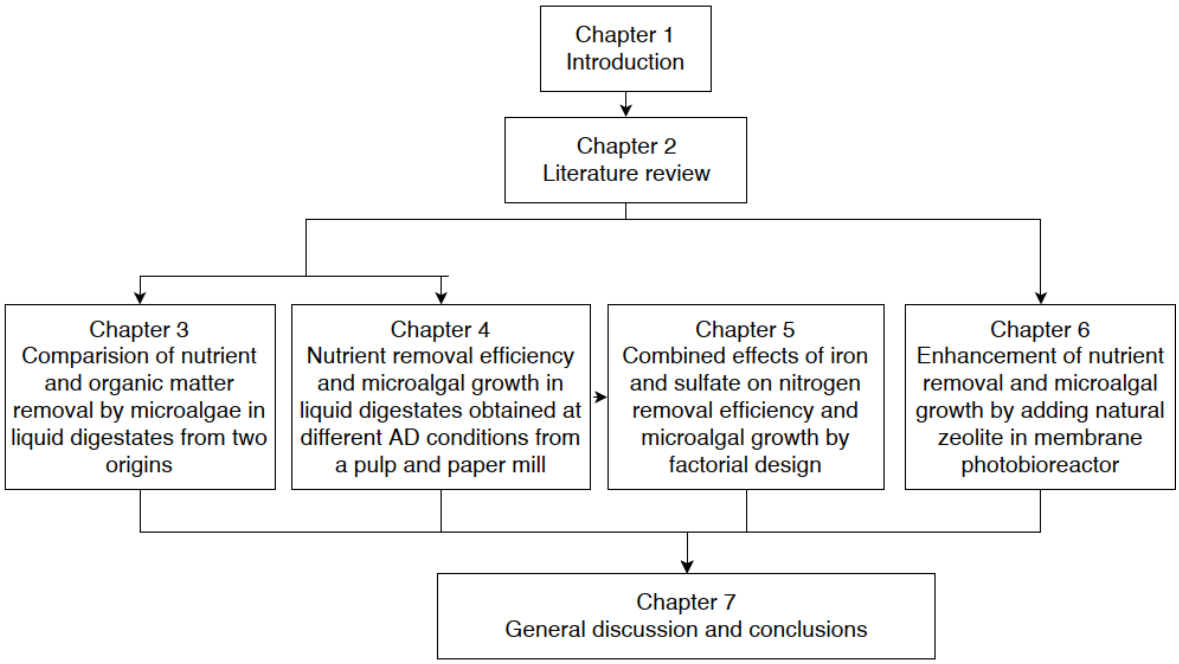



**Figure 1.1** Overview of the structure of this PhD thesis

## References


Abinandan, S., Shanthakumar, S., 2015. Challenges and opportunities in application of microalgae (*Chlorophyta*) for wastewater treatment: a review. Renewable and Sustainable Energy Reviews, 52, 123-132.

Ahluwalia, S.S., Goyal, D., 2007. Microbial and plant derived biomass for removal of heavy metals from wastewater. Bioresource Technology, 98(12), 2243-2257.

Andersen, R.A., 1992. Diversity of eukaryotic algae. Biodiversity and Conservation, 1(4), 267-292.

Beuckels, A., Smolders, E., Muylaert, K., 2015. Nitrogen availability influences phosphorus removal in microalgae-based wastewater treatment. Water Research, 77, 98-106.

Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: status and prospects. Renewable and Sustainable Energy Reviews, 19, 360-369.

Chernicharo, C.A.L., Van Lier, J.B., Noyola, A., Ribeiro, T.B., 2015. Anaerobic sewage treatment: state of the art, constraints and challenges. Reviews in Environmental Science and Bio/Technology, 14(4), 649-679.

Clarens, A.F., Resurreccion, E.P., White, M.A., Colosi, L.M., 2010. Environmental life cycle comparison of algae to other bioenergy feedstocks. Environmental Science & Technology, 44(5), 1813-1819.

Coppens, J., Grunert, O., Van Den Hende, S., Vanhoutte, I., Boon, N., Haesaert, G., De Gelder, L., 2016. The use of microalgae as a high-value organic slow-release fertilizer results in tomatoes with increased carotenoid and sugar levels. Journal of Applied Phycology, 28(4), 2367-2377.

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 Research, 38(2), 466-474.

Di Caprio, F., Altimari, P., Pagnanelli, F., 2018. Integrated microalgae biomass production and olive mill wastewater biodegradation: Optimization of the wastewater supply strategy. Chemical Engineering Journal, 349, 539-546.

Kacprzak, M., Neczaj, E., Fijałkowski, K., Grobelak, A., Grosser, A., Worwag, M., Rorat, A., Brattebo, H., Almås, Å., Singh, B.R., 2017. Sewage sludge disposal strategies for sustainable development. Environmental Research, 156, 39-46.

Kumar, A., Kumar, N., Baredar, P., Shukla, A., 2015. A review on biomass energy resources, potential, conversion and policy in India. Renewable and Sustainable Energy Reviews, 45, 530-539.

Mane, P.C., Bhosle, A.B., 2012. Bioremoval of Some Metals by Living Algae *Spirogyra* sp. and *Spirullina* sp. from aqueous solution. International Journal of Environmental Research, 6(2), 571-576.

Mehta, C.M., Khunjar, W.O., Nguyen, V., Tait, S., Batstone, D.J., 2015. Technologies to recover nutrients from waste streams: a critical review. Critical Reviews in Environmental Science and Technology, 45(4), 385-427.

Melvin, S.D., Leusch, F.D., 2016. Removal of trace organic contaminants from domestic wastewater: A meta-analysis comparison of sewage treatment technologies. Environment International, 92, 183-188.

Richmond, A. ed., 2004. Handbook of microalgal culture: biotechnology and applied phycology (Vol. 577). Oxford: Blackwell Science.

Sun, C.H., Fu, Q., Liao, Q., Xia, A., Huang, Y., Zhu, X., Reungsang, A., Chang, H.X., 2019. Life-cycle assessment of biofuel production from microalgae via various bioenergy conversion systems. Energy.

Tanabe, Y., Nishibayashi, Y., 2013. Developing more sustainable processes for ammonia synthesis. Coordination Chemistry Reviews, 257(17-18), 2551-2564.

Tchobanoglous, G., Stensel, H.D., Tsuchinashi, R., Burton, F., Abu-Orf, M., Bowden, G., Pfrang, W., 2014. Wastewater engineering treatment and reuse. New York, US: McGraw-Hill Education.

Van Drecht, G., Bouwman, A.F., Harrison, J., Knoop, J.M., 2009. Global nitrogen and phosphate in urban wastewater for the period 1970 to 2050. Global Biogeochemical Cycles, 23(4).

Xia, A., Murphy, J.D., 2016. Microalgal cultivation in treating liquid digestate from biogas systems. Trends in Biotechnology, 34(4), 264-275.

# 2 Microalgae and their use in wastewater treatment

## 2.1 Microalgae and their applications

Microalgae are generally defined as microscopic organisms, which can carry out photosynthetic activities (Richmond, 2004). Oxygen production of the Earth's atmosphere largely depends on photosynthesis, a process used by living organisms to convert light energy and $CO_2$ to chemical energy in form of organic compounds (Bryant and Frigaard, 2006). Microalgae can be divided into prokaryotic (cyanobacteria) and eukaryotic microorganisms (e.g. green algae and diatoms) (Wijffels et al., 2013). Microalgae are present in both aquatic and terrestrial ecosystems including lakes, ponds, soil, rocks, ice and snow (Andersen, 1992). The exact number of algal species is not known as the biodiversity of algae is enormous (Guiry, 2012). The estimated number of living algae (macroalgae and microalgae) has varied from 30,000 to over 1 million, while a conservative estimation on total number of algal species according to Guiry (2012) is approximately 72,500. Pure cultures of different microalgae have been isolated and maintained in many countries. For example, University of Coimbra (Portugal) is considered to have one of the world's largest microalgal culture collections with more than 4000 strains and 1000 species (Mata et al., 2010). Goettingen University (SAG, Germany), the University of Texas Algal Culture Collection (USA), and the National Institute for Environmental Studies Collection (Japan) are also well-known collections of algal cultures with more than 2000 strains in each of them (Mata et al., 2010).

In the early 1950s, microalgal biomass was considered to be one of the potential candidates as an alternative protein source for human and animal nutrition due to the predictions of an insufficient protein supply for the growing human population (Spolaore et al., 2006). Nowadays, microalgae are

studied and used for different applications such as human nutrition, animal feed, cosmetics, pigments, pharmaceuticals, fertilizers, energy and fuels, $CO_2$ mitigation and wastewater treatment (for a review, see e.g. Rizwan et al., 2018).

In microalgal biotechnology, monocultures are typically used for experimental and demonstration purposes as well as in commercial applications because specific microalgal species have certain desired characteristics. For example, *Spirulina*, which naturally grows in lakes, has been used as food or food supplement for hundreds or even thousands of years due to its health promoting and pharmacological properties such as high content of protein and edible fiber (Liang et al., 2004). *Dunaliella salina* and *Haematococcus pluvialis* have become the commercial sources of high-value products as they can accumulate high contents of β-carotene and astaxanthin, respectively (Hosseini Tafreshi and Shariati, 2009; Lorenz and Cysewski, 2000). Many studies have also been carried out to integrate microalgal cultivation with wastewater treatment (Coppens et al., 2016; Di Caprio et al., 2018; He et al., 2013; Polishchuk et al., 2015). It has been shown that for example *Chlorella* (Marjakangas et al., 2015), *Scenedesmus* (Jia et al., 2016) and *Spirulina* (Phang et al., 2000) species can remove e.g. nutrients, organic matter and heavy metals from wastewaters.

In reality, monocultures may be difficult to maintain in open systems due to susceptibility to contamination by wild algal strains, grazers, and bacteria (Carney et al., 2016). In case of wastewater treatment applications, the use and maintenance of axenic monocultures is impossible due to potential presence of microorganisms in incoming wastewaters and because outdoor open pond facilities are often considered preferable for practical scale applications (Rawat et al., 2011). Thus, using microalgal polycultures with two or more species exhibiting mutualistic or neutralistic relationships can be an effective way to enhance wastewater treatment efficiency and biomass production as different microalgae can utilize the cultivation environment differently to promote nutrient uptake rates and compete with other microorganisms (Cardinale, 2011; Stockenreiter et al., 2016).

### 2.1.1 Microalgal growth requirements with respect to wastewaters

Efficient cultivation of microalgae requires optimization of several parameters including temperature, pH, carbon source and nutrient availability, N/P ratio, light conditions and mixing (for reviews, see Hwang et al., 2016 and Lakaniemi, 2012). Carbon, nitrogen and phosphorus are three essential elements required in significant quantities for microalgal growth, whilst small concentrations of other elements such as iron, magnesium, sulfur, and potassium are also needed (Cai et al., 2013).

Light is essential for photosynthetic growth and light sources can be divided into sunlight and artificial light. Sunlight is a free, naturally available light source while artificial light can provide more easily

adjustable and flexible light intensity and wavelength distribution to reach optimum light for the specific microalgal species of interest, as the optimum light conditions can vary widely among different microalgal genera and species (Singh and Singh, 2015). For example, the highest specific growth rate of *Chlorella minutissima* was obtained at light intensity ranging from 115 to 135 µmol photos $m^{-2}$ $s^{-1}$ while higher and lower light intensities decreased the growth rate (Aleya et al., 2011). The incident light intensity is often measured from the culture surface, but constant and sufficient light availability is hard to maintain throughout the cultivation system due to self-shading of microalgal cells (Xia and Murphy, 2016), whereas too high light intensity can cause photoinhibition (Lundquist et al., 2010). Cultivation of microalgae in wastewaters further complicates the optimization of light availability, as suspended solids present in many wastewaters may contribute to a high turbidity and reduce light availability for the microalgal cells inside the cultivation systems (Franchino et al., 2013; Xia and Murphy, 2016).

Most microalgae have optimum growth temperatures ranging from 22 to 35 °C (Singh and Singh, 2015). In general, higher and lower temperatures reduce microalgal growth rate (Aleya et al., 2011; Singh and Singh, 2015). Thus, microalgal cultivation systems used in practical wastewater treatment may need cooling or heating to optimize cultivation conditions especially if wastewaters and exhaust gases have extremely low or high temperatures (Hanagata et al., 1992; Lettinga et al., 2001). Apart from adjusting the temperatures of wastewater and exhaust gases, using of psychrophilic microalgae such as *Chlamydomonas pulsatilla* (Hulatt et al., 2017) and thermotolerant microalgae such as certain *Chlorella* spp. e.g. strain K35 (Hanagata et al., 1992) can be alternative options.

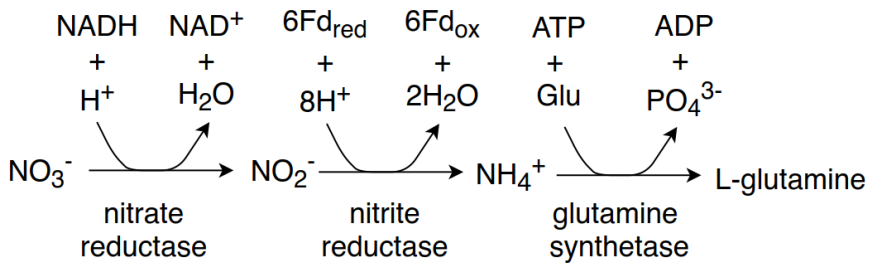
Mixing plays an important role in microalgal cultivations as efficient mixing can reduce cell sedimentation and enable efficient mass transfer and even light penetration in the entire culture volume (Carlozzi, 2003; Pruvost et al., 2006). However, mixing should not be high enough to damage the cells (Miron et al., 1999). Mixing can be carried out in several ways depending on the cultivation system: e.g. using mechanical agitators, mechanical pumps and gas sparging (Norsker et al., 2011).

Most microalgae can utilize organic (e.g. glucose and acetate) and inorganic ($CO_2$ and carbonate salts) carbon sources for growth (El Baky et al., 2012; Kim et al., 2013; Wright and Hobbie, 1966). In fact, most wastewaters contain organic carbon, which can support heterotrophic (organic carbon as carbon source) or mixotrophic (organic carbon and inorganic carbon from atmosphere or exhaust gases) growth of microalgae (Chen et al., 2011; He et al., 2013; Jaatinen et al., 2016). $CO_2$ from atmosphere or exhaust gases is often used in microalgal cultivation to enable photoautotrophic or mixotrophic growth (Cheah et al., 2015). Some microalgae such as marine microalga *Chlorococcum littorale* can tolerate up to 40–60% $CO_2$ concentrations (Kodama, 1993) because its photosystem II is protected from photoinhibition by keeping the chloroplastic pH constant (Iwasaki et al., 1998).

However, the high $CO_2$ concentration might not result in high microalgal $CO_2$ uptake efficiency if long enough $CO_2$ retention time is not provided (Judd et al., 2015; Lakaniemi et al., 2015).

Nitrogen is usually supplied to microalgae as nitrate, ammonium or urea (Cai et al., 2013; Hulatt et al., 2012). Nitrate is the most thermodynamically stable form of nitrogen in oxidized aquatic environments because nitrate is more oxidized than ammonium and urea (Barsanti and Gualtieri, 2014). In wastewaters, a mixture of different nitrogen sources can also be available for microalgal growth (De-Bashan et al., 2004). Nitrogen in the microalgal cells exists as organic nitrogen, and the process to convert inorganic nitrogen to organic form is known as assimilation (Cai et al., 2013). Ammonium can be directly assimilated into amino acids using glutamine synthetase, but nitrate and nitrite have to first undergo reduction to ammonium with the assistance of nitrate reductase and nitrite reductase, respectively (Figure 1A) (Cai et al., 2013). Thus, ammonium is thought to be the preferred form of nitrogen for microalgae as its assimilation requires less energy (Cai et al., 2013). It has been reported that the presence of ammonium up to certain concentration, which was dependent on species, could reduce nitrate uptake rate by algae (Maestrini et al., 1986). However, high concentrations of ammonium can be toxic to microalgal growth especially at alkaline conditions when it exists as ammonia (Abeliovich and Azov, 1976). Ammonia can also evaporate easily at high temperature and pH (Emerson et al. 1975; Zimmo et al. 2003). For utilization of urea (Figure 1B), microalgae can use both urease (urea amidohydrolase) and ATP-urea amidolyase (UALase) to catabolize urea to $NH_3$ and $CO_2$ (Naylor, 1970; Roon and Levenberg, 1968). Produced $NH_3$ can react with water to form $NH_4^+$, which is available for microalgal growth (Davis et al., 1953; Jaatinen et al., 2016). Hulatt et al. (2012) reported that biomass concentration of both *Chlorella vulgaris* and *Dunaliella tertiolecta* was slightly higher in a synthetic medium with urea than with nitrate as nitrogen source. The lowest microalgal biomass concentration of *D. tertiolecta* cultivation was obtained in the medium with ammonium, while *C. vulgaris* did not survive with ammonium likely due to low pH in the cultures caused by low buffering capacity of the used medium (Hulatt et al., 2012).

A




B

$$CO(NH_2)_2 + H_2O \xrightarrow{urease} CO_2 + 2NH_3$$

$$Urea + ATP \xrightarrow[Mg^{2+},\ K^{+}]{} 2NH_3 + CO_2 + ADP + Pi$$

**Figure 2.1** Microalgal assimilation of inorganic nitrogen (A) and reactions of urea transformation to ammonia with the catalytic activity of urease and ATP-urea amidolyase (UALase) (B). Adapted from Cai et al. (2013) and Naylor (1970).

Phosphorus is another key nutrient required for microalgal growth as it is involved in energy transfer ($ADP + P_i \xrightarrow{Energy} ATP$) and in synthesis of cellular constituents such as phospholipids and nucleic acids (Miyachi et al., 1964). Based on an approximate microalgal biomass molecular formula $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$, much less phosphorus than nitrogen is required by microalgae (Chisti, 2008). However, luxury uptake of phosphorus has been shown to occur during microalgal growth when plenty of phosphorus is available, while the microalgae can utilize the stored additional phosphorus as internal resource when the availability of external phosphorus is not sufficient for growth (Kuhl, 1974). Phosphorus can be removed from wastewaters by microalgal assimilation (growth and luxury uptake) and/or chemical precipitation with e.g. calcium (Brown and Shilton, 2014; Shelef et al., 1984). However, it is difficult to quantitatively determine the assimilated and precipitated fractions of the removed phosphorus and these have typically not been differentiated in the scientific literature. These analyses could be considered in the future to provide more in-depth understanding of the pathways occurring during phosphorus removal by microalgae.

The effects of trace elements such as sulfur, iron, and magnesium on microalgal growth have also been studied to enhance e.g. microalgal biomass and lipid production (Gorain et al., 2013; Mera et al., 2016; Singh et al., 2015). For example, Lv et al. (2017) reported that higher *Chlorococcum* sp. GD biomass concentration was obtained in a synthetic medium with sulfate (18–271 mg $L^{-1}$) than without sulfate. The total lipid content (56.6% of dry biomass) in *Chlorella vulgaris* cultures with 0.67 mg $L^{-1}$ $Fe^{3+}$ was 3–7-fold higher than that in the media with lower iron concentrations (Liu et al.,

2008). Apart from utilizing trace elements to promote growth via active cellular uptake, metals can also be removed by some living microalgae from wastewaters via passive biosorption to cells' surface (Kaduková and Virčíková, 2005; Mane and Bhosle, 2012). As metals are toxic to most microalgae at high concentrations, non-living algae biomass has been studied for removal of e.g. copper and lead from aqueous solutions by biosorption (Deng et al., 2006; Hamdy, 2000).

Many microalgae prefer to grow in slightly alkaline conditions with a pH range of 7 to 9 (Pahazri et al., 2016). The optimum pH for *Dunaliella salina* is even between 9 and 11 (Hosseini Tafreshi and Shariati, 2009). However, flocculation of microalgae often happens at a high pH due to chemical precipitation of calcium and/or magnesium salts as well as ammonium stripping from the culture (Emerson et al. 1975; Shelef et al., 1984; Zimmo et al. 2003). Conversely, acidophilic microalgae such as *Euglena gracilis* can survive under extremely low pH conditions (e.g. 2.5–3.5) (Johnson, 2012; Yamane et al., 2001). It is generally known that the uptake of $NO_3^-$ and $NH_4^+$ increases and decreases pH, respectively (Goldman and Brewer, 1980). In addition, $CO_2$ and/or chemical additives can be added automatically to maintain the culture pH at suitable level for the specific microalgal species (Pahazri et al., 2016).

### 2.1.2 Cultivation systems and downstream processing of microalgal cultivation

Microalgal cultivation systems can be divided into three main categories: open systems, closed systems, and hybrid systems (for a review, see e.g. Cai et al., 2013). Open systems are generally natural or artificial shallow ponds or simple open tanks, closed systems are transparent vessels known as photobioreactors that can be built in different shapes and sizes, and hybrid systems are systems that integrate open pond(s) and closed photobioreactor(s) in one cultivation system (Abinandan and Shanthakumar, 2015; Cai et al., 2013; Lakaniemi, 2012). To enable photosynthetic growth of microalgae, efficient illumination is usually required for microalgal cultivation systems, which makes the different compared to bioreactors used for cultivation of heterotrophic organisms (Eriksen, 2008). In addition to requiring an external light source, raceway ponds, which typical open systems for microalgal cultivation, are typically shallow (e.g. 0.15-0.3 m) to provide enough light for microalgal growth (Arbib et al., 2017; Cai et al., 2013). The typical closed systems include tubular, flat plate, and column photobioreactors, which are made of transparent materials and designed to have short light paths (e.g. tube and column diameter: 0.1–0.4 m) for efficient light penetration (for reviews, see e.g. Cai et al., 2013; Lakaniemi, 2012).

Open systems have relatively low construction and operation costs, but they typically enable low biomass productivity and require large land areas (Chinnasamy et al., 2010; Huo et al., 2012; Miron et al., 1999). Closed photobioreactors can be designed to increase photosynthetic efficiency and enable more controlled conditions for biomass production (Chinnasamy et al., 2010). However, they

are more expensive to build and operate than open systems and difficult to scale up (Chinnasamy et al., 2010; Miron et al., 1999). Currently, most commercial scale algal cultivation systems are open ponds (Abinandan and Shanthakumar, 2015). Some microalgal strains (e.g. *Haematococcus pluvialis*) typically used for high-value products in food supplement, cosmetics and pharmaceutical industries require growth environment free of competing microorganisms and are therefore cultivated in closed systems as the high value of the recovered product makes the overall process economical (Lorenz and Cysewski, 2000). Hybrid systems can consist of e.g. two stages, where closed photobioreactors are used for sufficient volume of cells under near-optimal growth conditions as the first stage and then open ponds are used for astaxanthin production under environmental and nutrient stress (Lorenz and Cysewski, 2000). In recent years, different cultivation systems have been combined and novel cultivation systems have been proposed in some studies to promote the performance of cultivation systems (e.g. microalgal biomass production and wastewater treatment efficiency) (Table 1). For example, biofilm carriers or sheets have been installed inside high-rate ponds to improve nutrient removal and microalgal yields compared to traditional high-rate ponds relying solely on activity of suspended cells (Gao et al., 2015; Lee et al., 2014). In addition, biofilm sheets could also be installed outside of high rate ponds as a hybrid system (de Assis et al., 2017). It has been shown that the microalgal production increased, however, the cost for operating and feasibility for scale-up of photobioreactors with novel configurations remains to be determined (Table 2.1).

**Table 2.1** Selected microalgal studies utilizing different hybrid and novel cultivation systems developed to enhance e.g. microalgal biomass production, wastewater treatment efficiency, or production of high-value compounds.

| Microalgal culture | Medium ($mg\ L^{-1}$) | Cultivation system (working volume) | Novelty (or hybrid nature) | Operation mode (time in days) | Average biomass productivity ($g\ L^{-1}\ d^{-1}$) | Nutrient removal efficiency (%) | Reference |
|---|---|---|---|---|---|---|---|
| *Chlorella vulgaris* | Modified BG11 | Fractal tree-like photobioreactor (10 L) | The fractal tree-like structure to provide high gas holdup and improve mass transfer | Batch (4) | 0.06 | n.a. | Zhao et al., 2019 |
| *Chlorella zofingiensis* | Artificial medium | Fermenter + Rotating floating photobioreactor (5 L) | Heterotrophic cultivation to achieve a high microalgal biomass concentration followed by astaxanthin induction process in a patented rotating floating photobioreactor | Fed-batch (14) + Batch (4) | 7.03 | n.a. | Zhang et al., 2017 |
| *Desmodesmus* sp. | Piggery biogas slurry ($NH_4$-N: 765, TP:37) | Flat-plate open photobioreactor (1 L) | Aeration and mixing achieved by rational influent mode (multi-point mode, sprinkling mode and underflow mode) to save costs | Continuous (10) | 0.47 | $NH_4$-N: 95 TP: 75 | Luo et al., 2019. |
| *Dunaliella salina* | Seawater desalination concentrate | Outdoor floating inflatable-membrane photobioreactor (75 L) | Floating offshore microalgal photobioreactors to reduce land use and utilize wave energy for mixing | Batch (12) | 0.03 | n.a. | Zhu et al., 2018 |
| *Haematococcus* | n.a. | Closed photobioreactors + Open ponds (500 000 L) | Closed and open systems combined in a two-stage cultivation system | Batch (5) | n.a. | n.a. | Lorenz and Cysewski, 2000 |
| *Nannochloris atomus* | Artificial seawater medium | Outdoor floating horizontal photobioreactor (65 L) | Closed horizontal raceway to reduce costs and contamination | Semi-continuous (165) | 18.2 $g\ m^{-2}\ d^{-1}$ | n.a. | Dogaris et al., 2015 |
| Mixed culture (dominated by *Chlorella vulgaris*) | Municipal wastewater ($NH_3$-N: 37 Soluble phosphorus: 5.2) | Outdoor hybrid high-rate pond (1 $m^3$) with biofilm reactor (surface area: 1.0 $m^2$) | Biofilm sheets and open system combined in a two-stage cultivation system for easy biomass harvesting | Continuous (~42) | 6.8 $g\ m^{-2}\ d^{-1}$ | $NH_3$-N: 84 Soluble phosphorus: 21 | de Assis et al., 2017 |

n.a.= not available

Some microalgal species such as *Chlorella protothecoides* and *Chlorella vulgaris* can be cultivated under dark conditions in fermenters using organic carbon as the energy and carbon source (Chen et al., 2011; Borowitzka, 2013). The costs of organic carbon sources and presence of bacterial contamination might have hindered the development of this type of microalgal cultivation systems (Chen et al., 2011). However, cost of the organic carbon source may not be a big concern when the heterotrophically cultivated biomass such as *Crypthecodinium*, *Schizochytrium* and *Ulkenia* is used to generate high-value products such as long-chain polyunsaturated fatty acids, which has been done in commercial scale (Borowitzka, 2013). In addition, heterotrophic growth of microalgae can be significant in wastewater treatment applications as most of waste streams contain organic carbon (He et al., 2013; Jaatinen et al., 2016).

Different operation modes including batch, fed-batch, semi-continuous, and continuous operation, have been used in all the three cultivation system types depending on the application and/or desired products. For example, *Haematococcus* has been batch-cultivated for natural astaxanthin production because accumulation of astaxanthin happens under environmental and nutrient stress, which are difficult to induce in continuous operation (Lorenz and Cysewski, 2000). Similarly, continuous-flow operation may not induce the production of lipids in the microalgal cells as lipids typically start to accumulate only under nitrogen starvation (Chen et al., 2011; Mujtaba et al., 2012). To date, batch mode has been used in most studies focusing on the use of microalgae for wastewater treatment (Di Caprio et al., 2018; He et al., 2013; Jia et al., 2016; Usha et al., 2016). Cultivations with different operation modes have so far been rarely compared in the scientific literature. However, higher nutrient removal efficiencies were obtained from wastewater by *Scenedesmus* sp. in continuous-flow reactors than in batch systems due to higher maximum growth rate obtained in continuous-flow mode (McGinn et al., 2012).

To use the generated microalgal biomass for production of energy, biofuels, fertilizers and/or other products, the biomass typically needs to be separated from the treated wastewater or cultivation media via a harvesting process. Microalgal harvesting is possible via mechanical (e.g. centrifugation and flotation), chemical (chemical coagulation/flocculation) and biological methods (auto-flocculation and bio-flocculation), or with combinations of these methods (for reviews, see e.g. Christenson and Sims, 2011; Pahazri et al., 2016). It is also common to combine two or more of these methods to enable an efficient and/or cost-effective harvesting (Pahazri et al., 2016). However, no single method appears to be feasible for all applications and microalgal species (Gerardo et al., 2015; Grima et al., 2003) as the microalgal cell size and final product strongly contribute to the selection of harvesting method(s) and required post-harvest processes such as drying and extraction of desired cellular components (for a review, see Pahazri et al., 2016). The water separated from the biomass during harvesting/post-harvest processing may be discharged directly to natural environment or sent for further use/treatment (Xia and Murphy, 2016).

## 2.2 Wastewaters and their treatments

Large amount of wastewaters are produced due to the rapid development of urbanization and fast growth of world population (Van Drecht et al., 2009). The composition of wastewaters is largely dependent on their source and they can contain many environmentally harmful substances such as suspended solids, biodegradable organics, nutrients, metals, and pathogens (Tchobanoglous et al., 2014). In general, the wastewaters can be categorized into three main types: municipal wastewaters, agricultural wastewaters, and industrial wastewaters. Municipal wastewaters are usually discharged from residential, commercial, and institutional areas (Tchobanoglous et al., 2014). Agricultural wastewaters are mainly produced from livestock operations and industrial wastewaters originate from a wide range of industries such as textile, food, pulp and paper, tannery, electroplating, mining, and mineral processing (Cai et al., 2013; Sophonsiri and Morgenroth, 2004; Thayalakumaran et al., 2003).

Agricultural wastewaters typically contain higher concentration of nutrients and organic matter compared to municipal wastewaters, while the compositions of industrial wastewaters are dependent on the industry (Ji et al., 2014; Sophonsiri and Morgenroth, 2004). For example, nutrient concentrations and COD of meat processing wastewater can vary from 20 to 2000 mg $L^{-1}$ while mining wastewaters can have less than 5 mg $L^{-1}$ nitrogen, phosphorus, and COD (Ji et al., 2014; Thayalakumaran et al., 2003; Touahria et al., 2016). In addition, the composition and generation volume of the same type of wastewater also vary with e.g. location and season. For example, diet change can result in different municipal wastewater composition between summer and winter likely due to the climates (Li and Lu, 2017).

Most of the wastewaters need treatment before being discharged to the environment due to the compounds that they contain. Certain physical characteristics of the wastewaters such as colors and odors often caused by various organic compounds can be directly noticed by the public and have harmful effects on the environment, and human and animal health (Tchobanoglous et al., 2014). In addition, eutrophication is caused by release of nitrogen and phosphorus and some human diseases are due to uptake of heavy metals, pathogens and other toxics (Tchobanoglous et al., 2014). In general, municipal wastewater is transported through sewage systems to a centralized wastewater treatment plant for treatment before being discharged to the environment. Agricultural and industrial wastewaters can be

treated with specific methods close to their generation source or transported to a centralized municipal wastewater treatment plant to be treated together with municipal wastewaters.

Typical wastewater treatment process includes physical, chemical and biological unit processes (Tchobanoglous et al., 2014). In a municipal wastewater treatment plant (Figure 2.2 A), the treatment process can consist of screening, grid removal, primary sedimentation, activated-sludge process, secondary sedimentation, and disinfection (Tchobanoglous et al., 2014). The sludge after primary and secondary sedimentation is typically sent to an anaerobic digester for stabilization and is used in agriculture as fertilizer or combusted to produce energy (Figure 2.2). The activated-sludge process consumes lots of energy as biodegradable organic matter and nutrients are removed by microbes with aeration (Tchobanoglous et al., 2014). Industrial wastewaters can contain high amount of e.g. COD, ammonia, and/or heavy metals (Ji et al., 2014; Thayalakumaran et al., 2003; Touahria et al., 2016). The traditional methods to remove heavy metals are e.g. chemical precipitation, adsorption, and ion exchange (Barakat, 2011).

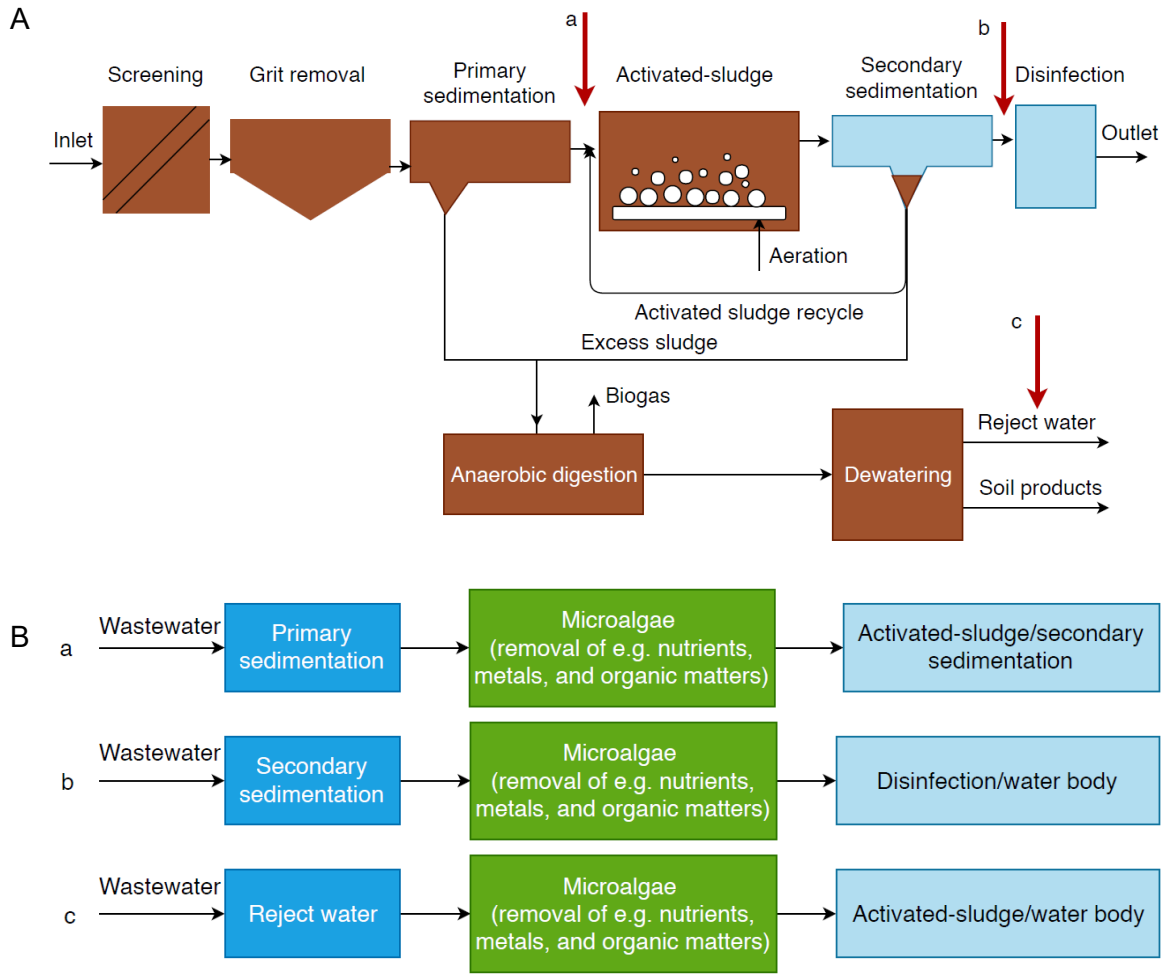



**Figure 2.2** A typical municipal wastewater treatment process (modified from Tchobanoglous et al., 2014) (A) and possible ways to integrate processes utilizing microalgae for treatment of wastewaters generated from (a) primary sedimentation, (b) secondary sedimentation, and (c) dewatering of activated sludge (B).

In recent years, it has been reported that microalgal treatment of wastewater provides a promising means to recover nutrients and metals from the wastewaters (Arias et al., 2018; El-Sheekh et al., 2005; He et al., 2013). The use of microalgae to remove e.g. COD and color from wastewaters has also been studied (Di Caprio et al., 2018; He et al., 2013; Wu et al., 2017). The advantages for microalgae use in wastewater treatment is the ability to simultaneously recover nitrogen and phosphorus, and generate algal biomass for e.g. energy, biofuel, and/or fertilizer production.

## 2.3 Microalgae in wastewater treatment

The studies focusing on cultivation of microalgae in wastewaters seem to have different three approaches: (1) microalgae are used in wastewater treatment to remove nutrients, metals and organic matter while microalgal biomass is produced as a by-product, (2) wastewater is used as a cheap nutrient source to produce microalgal biomass or specific biomass composition while efficient wastewater treatment is not in the focus, and (3) a compromise between microalgal biomass production and wastewater treatment has been sought to enable both treatment of wastewater and microalgal biomass production. The first approach will be mainly presented and discussed in the following paragraphs as that is most closely related to the aims of this thesis. Further information on the other two approaches can be from recent review papers (Chen et al., 2015; Christenson and Sims, 2011; Hwang et al., 2016).

The use of microalgae to treat various types of wastewaters (municipal, agricultural, and industrial) has been increasingly studied. The studies on nutrient recovery and organic matter removal by microalgae have mostly focused on municipal wastewaters due to their wide availability and non-inhibitory nutrient concentrations (for a review, see Cai et al., 2013). Municipal wastewaters are suitable for microalgal growth due to low ammonium concentrations to reduce the costs for pretreatments such as dilution (Abeliovich and Azov, 1976). For example, *Chlorella vulgaris* has been shown to remove 97% ammonium, 98% phosphorus and 26% DOC from municipal wastewater (total nitrogen: 29–174 mg $L^{-1}$) and more than 44–64% of TN in the wastewater was recovered into the microalgal biomass (He et al., 2013). Agricultural wastewaters being mainly produced from livestock operations e.g. animal manure effluents contain large amounts of nitrogen and phosphorus (for a review, see Cai et al., 2013). Microalgal cultivation in animal manure effluents has become an alternative to the current land application due to less requirement for transportation because the microalgal cultivation can be carried out near the effluent source (for a review, see Sheets et al., 2015). However, the high amount of suspended solids present in many agricultural wastewaters are not beneficial for microalgal growth due to the possible light limitation (Nam et al., 2017; Zhu et al., 2013).

Microalgae use for treatment of industrial wastewaters has been studied to remove e.g. heavy metals (Ahluwalia and Goyal, 2007), COD (Phang et al., 2000), and color (Lim et al., 2010). Conventional treatment processes such as chemical precipitation and ion exchange

are mostly applied to remove heavy metals from wastewaters (for a review, see Fu and Wang, 2011). Microalgal biosorption for heavy metal removal/recovery has been developed due to the drawbacks such as chemical addition and high sensitivity to pH of the conventional methods (Ayangbenro and Babalola, 2017; Barakat, 2011). For example, when 11 microalgal species were tested in batch digestion tubes, the zinc removal efficiencies were higher than the nickel removal efficiencies, which was likely due to higher microalgal affinity to zinc ions, and *Scenedesmus quadricauda* was the most effective species as it removed more than 99% nickel and zinc within 2 h (Chong et al., 2000). However, some industrial wastewaters may contain high concentrations of toxic compounds and low levels of N and P, which reduce the microalgal metal removal efficiency. Thus, compared to other wastewaters, some industrial wastewaters may not be competitive for microalgal cultivation due to low growth rate (for a review, see Umamaheswari and Shanthakumar, 2016). In addition, the produced microalgal biomass could need further treatment prior to the production of e.g. fertilizer and biodiesel because the high concentration of e.g. heavy metals, pharmaceuticals and/or dyes in the algal biomass, are harmful to the receiving environment or catalysts needed for biodiesel production (Viarengo, 1985; Wang et al., 2016).

Microalgal treatment could fully replace traditional wastewater treatment process or microalgae can be integrated with traditional wastewater treatment processes by replacing one or several of the existing unit processes used in a typical wastewater treatment plant (Figure 2.3A). For example, the microalgal treatment of wastewater pretreated by primary sedimentation can replace the activated-sludge process, if nutrients and organic matter are efficiently removed by the microalgae (Figure 2.3B-a). Alternatively, microalgal treatment can be added between the primary sedimentation and the activated-sludge unit in the existing process to reduce the aeration requirement of the activated-sludge process due to partial nutrient and organic matter removal/recovery by microalgae (Figure 2.3B-a). It has also been suggested that microalgal cultivation can be used to remove the residual nutrients and organic matter from traditionally treated wastewater e.g. after second sedimentation (Figure 2.3B-b). Microalgal cultivation can also be used to treat the wastewaters generated from dewatering of digestion of the excess sludge generated during traditional wastewater treatment (Figure 2.3B-c). Use of microalgae for wastewater treatment would require that the grown microalgal biomass can be harvested efficiently and thus separated from the treated water. Membrane photobioreactors might be one option for wastewater treatment as they enable separation of the biomass and liquid during operation (Gao et al., 2018). Last but not least, how and where to install the microalgal treatment is dependent on the overall costs

and added benefits of the modified treatment process compared to the existing process. It has been shown that efficiency of municipal wastewater treatment via microalgal may vary due to different compositions of wastewaters (Gentili, 2014; Wang et al., 2010). For example, the highest removal efficiencies (TN: 82.8%; $PO_4$-P: 85.6%; COD: 83.0%) were obtained by *Chlorella* sp. batch cultivation in the centrate generated from a sludge centrifuge due to sufficient concentration of nutrients (TN: 130 mg $L^{-1}$; TP: 200 mg $L^{-1}$) compared to wastewaters generated before primary setting, after primary, and from aeration tank in a municipal wastewater treatment plant (Wang et al., 2010).

Some studies have combined wastewaters obtained from different sources (municipal, agricultural, and industrial) or different points of treatment processes of one source (e.g. municipal wastewater treatment plant) to achieve the optimal conditions for nutrient and pollutant removal and microalgal growth (Bohutskyi et al., 2016; Gentili, 2014). The main benefit of combining wastewaters is to optimize the composition (e.g. pH, nutrient concentration, turbidity, and N/P ratio) to provide suitable conditions for microalgal growth. However, in practice it could be difficult to combine wastewaters from different sources (municipal, agricultural, and industrial) due to typically long distances between locations where these different wastewaters are generated. In the future, more studies are needed simultaneously considering e.g. wastewater treatment efficiency, microalgal biomass yield, and operating cost to decide if the microalgae use in wastewater treatment should replace conventional treatment process or be used as an additional processing step.

In waste streams generated from anaerobic digestion also known as liquid digestates or reject waters, ammonium nitrogen is the major component of total nitrogen (Posadas et al., 2016; Wang et al., 2010). Therefore, selection of liquid digestate dilution has played an important role to ensure the microalgal growth, because high ammonium concentration can limit microalgal growth (Abeliovich and Azov, 1976). In addition, microalgal growth limitation by high turbidity of liquid digestates can be also reduced/avoided by dilution (Akhiar et al., 2017). For example, *Chlorella vulgaris* was shown to efficiently remove ammonium and phosphate (>96%) from 10-times diluted liquid digestates of cattle slurry and raw cheese whey but did not survive in the undiluted and 2-times diluted liquid digestates likely due to high turbidity of the medium (Franchino et al., 2013). Technically dilution is not a problem, as part of the treated water could be recycled to the process or another type of wastewater with low turbidity could be used to dilute the liquid digestate. However, cost of treatment system grows with increased treated water volume due to dilution. In addition to decreaing turbidity, N/P ratios can be adjusted to the optimal range for nutrient removal and microalgal

growth by using a mixture of wastewaters. When *Chlorella vulgaris* was cultivated in domestic wastewater with various N/P ratios ranging from 0 to 80, TN removal efficiency remained stable at all studied N/P ratios, but TP removal efficiency had a decreasing trend with the increasing N/P ratio (Choi and Lee, 2015). This means that TP removal efficiency can be increased by using proper N/P ratios, which are dependent on the species and growth conditions (Klausmeier et al., 2004; Xin et al., 2010). In addition to microalgae use in wastewater treatment, it has been proposed that microalgae could be used to capture $CO_2$ from biogas (Xia and Murphy, 2016).

To improve the system performance of microalgae in wastewater treatment, additives such as zeolite (Markou et al., 2014) and activated carbon (Kuo, 2017) have been studied with the aim to enhance microalgal growth and removal of nutrients and other pollutants. For example, 50 g $L^{-1}$ zeolite addition to a membrane photobioreactor with shock loadings was observed to enhance the microalgal-bacterial system stability, pollutant removal efficiency, and biomass concentration (Wang et al., 2018). Zeolite acted as adsorbent during the ammonium shock loadings and likely provided microorganism habitats to form biofilms (Wang et al., 2018). However, the additives to the system should be carefully examined before being applied due to potential drawbacks to the final products and the nature environment.

## References


Abeliovich, A.H.A.R.O.N., Azov, Y., 1976. Toxicity of ammonia to algae in sewage oxidation ponds. Applied and Environmental Microbiology, 31(6), 801-806.

Abinandan, S., Shanthakumar, S., 2015. Challenges and opportunities in application of microalgae (*Chlorophyta*) for wastewater treatment: a review. Renewable and Sustainable Energy Reviews, 52, 123-132.

Ahluwalia, S.S., Goyal, D., 2007. Microbial and plant derived biomass for removal of heavy metals from wastewater. Bioresource Technology, 98(12), 2243-2257.

Akhiar, A., Battimelli, A., Torrijos, M., Carrere, H., 2017. Comprehensive characterization of the liquid fraction of digestates from full-scale anaerobic co-digestion. Waste Management, 59, 118-128.

Aleya, L., Dauta, A., Reynolds, C.S., 2011. Endogenous regulation of the growth-rate responses of a spring-dwelling strain of the freshwater alga, *Chlorella minutissima*, to light and temperature. European Journal of Protistology, 47(4), 239-244.

Andersen, R.A., 1992. Diversity of eukaryotic algae. Biodiversity and Conservation, 1(4), 267-292.

Arbib, Z., de Godos, I., Ruiz, J., Perales, J.A., 2017. Optimization of pilot high rate algal ponds for simultaneous nutrient removal and lipids production. Science of the Total Environment, 589, 66-72.

Arias, D.M., Sole-Bundo, M., Garfi, M., Ferrer, I., Garcia, J., Uggetti, E., 2018. Integrating microalgae tertiary treatment into activated sludge systems for energy and nutrients recovery from wastewater. Bioresource Technology, 247, 513-519.

Ayangbenro, A., Babalola, O., 2017. A new strategy for heavy metal polluted environments: a review of microbial biosorbents. International Journal of Environmental Research and Public Health, 14(1), 94.

Barakat, M.A., 2011. New trends in removing heavy metals from industrial wastewater. Arabian Journal of Chemistry, 4(4), 361-377.

Barsanti, L., Gualtieri, P., 2014. Algae: anatomy, biochemistry, and biotechnology. CRC Press.

Bohutskyi, P., Kligerman, D.C., Byers, N., Nasr, L.K., Cua, C., Chow, S., Su, C., Tang, Y., Betenbaugh, M.J., Bouwer, E.J., 2016. Effects of inoculum size, light intensity, and dose of anaerobic digestion centrate on growth and productivity of *Chlorella* and *Scenedesmus*

microalgae and their poly-culture in primary and secondary wastewater. Algal Research, 19, 278-290.

Borowitzka, M.A., 2013. High-value products from microalgae—their development and commercialisation. Journal of Applied Phycology, 25(3), 743-756.

Brown, N., Shilton, A., 2014. Luxury uptake of phosphorus by microalgae in waste stabilisation ponds: current understanding and future direction. Reviews in Environmental Science and Bio/Technology, 13(3), 321-328.

Bryant, D.A., Frigaard, N.U., 2006. Prokaryotic photosynthesis and phototrophy illuminated. Trends in Microbiology, 14(11), 488-496.

Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: status and prospects. Renewable and Sustainable Energy Reviews, 19, 360-369.

Cardinale, B.J., 2011. Biodiversity improves water quality through niche partitioning. Nature, 472(7341), 86.

Carlozzi, P., 2003. Dilution of solar radiation through “culture” lamination in photobioreactor rows facing south–north: a way to improve the efficiency of light utilization by cyanobacteria (*Arthrospira platensis*). Biotechnology and Bioengineering, 81(3), 305-315.

Carney, L.T., Wilkenfeld, J.S., Lane, P.D., Solberg, O.D., Fuqua, Z.B., Cornelius, N.G., Gillespie, S., Williams, K.P., Samocha, T.M., Lane, T.W., 2016. Pond crash forensics: presumptive identification of pond crash agents by next generation sequencing in replicate raceway mass cultures of *Nannochloropsis salina*. Algal Research, 17, 341-347.

Cheah, W.Y., Show, P.L., Chang, J.S., Ling, T.C., Juan, J.C., 2015. Biosequestration of atmospheric $CO_2$ and flue gas-containing $CO_2$ by microalgae. Bioresource Technology, 184, 190-201.

Chen, C.Y., Yeh, K.L., Aisyah, R., Lee, D.J., Chang, J.S., 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. Bioresource Technology, 102(1), 71-81.

Chen, G., Zhao, L., Qi, Y., 2015. Enhancing the productivity of microalgae cultivated in wastewater toward biofuel production: a critical review. Applied Energy, 137, 282-291.

Chinnasamy, S., Bhatnagar, A., Claxton, R., Das, K.C., 2010. Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium. Bioresource Technology, 101(17), 6751-6760.

Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends in Biotechnology, 26(3), 126-131.

Choi, H.J., Lee, S.M., 2015. Effect of the N/P ratio on biomass productivity and nutrient removal from municipal wastewater. Bioprocess and Biosystems Engineering, 38(4), 761-766.

Christenson, L., Sims, R., 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnology Advances, 29(6), 686-702.

Chong, A.M.Y., Wong, Y.S., Tam, N.F.Y., 2000. Performance of different microalgal species in removing nickel and zinc from industrial wastewater. Chemosphere, 41(1-2), 251-257.

Coppens, J., Grunert, O., Van Den Hende, S., Vanhoutte, I., Boon, N., Haesaert, G., De Gelder, L., 2016. The use of microalgae as a high-value organic slow-release fertilizer results in tomatoes with increased carotenoid and sugar levels. Journal of Applied Phycology, 28(4), 2367-2377.

Davis, E.A., Dedrick, J., French, C.S., Milner, H.W., Myers, J., Smith, J.H.C., Spoehr, H.A., 1953. Laboratory experiments on *Chlorella* culture at the Carnegie Institution of Washington Department of plant biology. Algal Culture from Laboratory to Pilot Plant, 105-53.

de Assis, L.R., Calijuri, M.L., do Couto, E.D.A., Assemany, P.P., 2017. Microalgal biomass production and nutrients removal from domestic sewage in a hybrid high-rate pond with biofilm reactor. Ecological Engineering, 106, 191-199.

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 Research, 38(2), 466-474.

Deng, L., Su, Y., Su, H., Wang, X., Zhu, X., 2006. Biosorption of copper (II) and lead (II) from aqueous solutions by nonliving green algae *Cladophora fascicularis*: equilibrium, kinetics and environmental effects. Adsorption, 12(4), 267-277.

Di Caprio, F., Altimari, P., Pagnanelli, F., 2018. Integrated microalgae biomass production and olive mill wastewater biodegradation: Optimization of the wastewater supply strategy. Chemical Engineering Journal, 349, 539-546.

Dogaris, I., Welch, M., Meiser, A., Walmsley, L., Philippidis, G., 2015. A novel horizontal photobioreactor for high-density cultivation of microalgae. Bioresource Technology, 198, 316-324.

El Baky, H.H.A., El-Baroty, G.S., Bouaid, A., Martinez, M., Aracil, J., 2012. Enhancement of lipid accumulation in *Scenedesmus obliquus* by optimizing $CO_2$ and $Fe^{3+}$ levels for biodiesel production. Bioresource Technology, 119, 429-432.

El-Sheekh, M.M., El-Shouny, W.A., Osman, M.E., El-Gammal, E.W., 2005. Growth and heavy metals removal efficiency of *Nostoc muscorum* and *Anabaena subcylindrica* in

sewage and industrial wastewater effluents. Environmental Toxicology and Pharmacology, 19(2), 357-365.

Emerson, K., Russo, R.C., Lund, R.E., Thurston, R.V., 1975. Aqueous ammonia equi-librium calculations: effect of pH and temperature. Journal of the Fisheries Board of Cana-da, 32(12), 2379-2383.

Franchino, M., Comino, E., Bona, F., Riggio, V.A., 2013. Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate. Chemosphere, 92(6), 738-744.

Fu, F., Wang, Q., 2011. Removal of heavy metal ions from wastewaters: a review. Journal of Environmental Management, 92(3), 407-418.

Gao, F., Peng, Y.Y., Li, C., Cui, W., Yang, Z.H., Zeng, G.M., 2018. Coupled nutrient removal from secondary effluent and algal biomass production in membrane photobioreactor (MPBR): effect of HRT and long-term operation. Chemical Engineering Journal, 335, 169-175.

Gao, F., Yang, Z.H., Li, C., Zeng, G.M., Ma, D.H., Zhou, L., 2015. A novel algal biofilm membrane photobioreactor for attached microalgae growth and nutrients removal from secondary effluent. Bioresource Technology, 179, 8-12.

Gentili, F.G., 2014. Microalgal biomass and lipid production in mixed municipal, dairy, pulp and paper wastewater together with added flue gases. Bioresource Technology, 169, 27-32.

Gerardo, M.L., Van Den Hende, S., Vervaeren, H., Coward, T., Skill, S.C., 2015. Harvesting of microalgae within a biorefinery approach: A review of the developments and case studies from pilot-plants. Algal Research, 11, 248-262.

Gorain, P.C., Bagchi, S.K., Mallick, N., 2013. Effects of calcium, magnesium and sodium chloride in enhancing lipid accumulation in two green microalgae. Environmental Technology, 34(13-14), 1887-1894.

Grima, E.M., Belarbi, E.H., Fernández, F.A., Medina, A.R., Chisti, Y., 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnology Advances, 20(7-8), 491-515.

Guiry, M.D., 2012. How many species of algae are there?. Journal of Phycology, 48(5), 1057-1063.

Hamdy, A.A., 2000. Removal of $Pb^{2+}$ by biomass of marine algae. Current Microbiology, 41(4), 239-245.

Hanagata, N., Takeuchi, T., Fukuju, Y., Barnes, D.J., Karube, I., 1992. Tolerance of microalgae to high $CO_2$ and high temperature. Phytochemistry, 31(10), 3345-3348.

He, P.J., Mao, B., Lü, F., Shao, L.M., Lee, D.J., Chang, J.S., 2013. The combined effect of bacteria and *Chlorella vulgaris* on the treatment of municipal wastewaters. Bioresource Technology, 146, 562-568.

Hosseini Tafreshi, A., Shariati, M., 2009. *Dunaliella* biotechnology: methods and applications. Journal of Applied Microbiology, 107(1), 14-35.

Hulatt, C.J., Berecz, O., Egeland, E.S., Wijffels, R.H., Kiron, V., 2017. Polar snow algae as a valuable source of lipids?. Bioresource Technology, 235, 338-347.

Hulatt, C.J., Lakaniemi, A.M., Puhakka, J.A., Thomas, D.N., 2012. Energy demands of nitrogen supply in mass cultivation of two commercially important microalgal species, *Chlorella vulgaris* and *Dunaliella tertiolecta*. BioEnergy Research, 5(3), 669-684.

Huo, S., Wang, Z., Zhu, S., Zhou, W., Dong, R., Yuan, Z., 2012. Cultivation of *Chlorella zofingiensis* in bench-scale outdoor ponds by regulation of pH using dairy wastewater in winter, South China. Bioresource Technology, 121, 76-82.

Hwang, J.H., Church, J., Lee, S.J., Park, J., Lee, W.H., 2016. Use of microalgae for advanced wastewater treatment and sustainable bioenergy generation. Environmental Engineering Science, 33(11), 882-897.

Iwasaki, I., Hu, Q., Kurano, N., Miyachi, S., 1998. Effect of extremely high-$CO_2$ stress on energy distribution between photosystem I and photosystem II in a 'high-$CO_2$'tolerant green alga, *Chlorococcum littorale* and the intolerant green alga *Stichococcus bacillaris*. Journal of Photochemistry and Photobiology B: Biology, 44(3), 184-190.

Jaatinen, S., Lakaniemi, A.M., Rintala, J., 2016. Use of diluted urine for cultivation of *Chlorella vulgaris*. Environmental Technology, 37(9), 1159-1170.

Ji, M.K., Kabra, A.N., Salama, E.S., Roh, H.S., Kim, J.R., Lee, D.S., Jeon, B.H., 2014. Effect of mine wastewater on nutrient removal and lipid production by a green microalga *Micratinium reisseri* from concentrated municipal wastewater. Bioresource Technology, 157, 84-90.

Jia, Q., Xiang, W., Yang, F., Hu, Q., Tang, M., Chen, C., Wang, G., Dai, S., Wu, H., Wu, H., 2016. Low-cost cultivation of *Scenedesmus* sp. with filtered anaerobically digested piggery wastewater: biofuel production and pollutant remediation. Journal of Applied Phycology, 28(2), 727-736.

Judd, S., van den Broeke, L.J., Shurair, M., Kuti, Y., Znad, H., 2015. Algal remediation of $CO_2$ and nutrient discharges: a review. Water Research, 87, 356-366.

Kaduková, J., Virčíková, E., 2005. Comparison of differences between copper bioaccumulation and biosorption. Environment International, 31(2), 227-232.

Kim, S., Park, J.E., Cho, Y.B., Hwang, S.J., 2013. Growth rate, organic carbon and nutrient removal rates of *Chlorella sorokiniana* in autotrophic, heterotrophic and mixotrophic conditions. Bioresource Technology, 144, 8-13.

Klausmeier, C.A., Litchman, E., Daufresne, T., Levin, S.A., 2004. Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. Nature, 429(6988), 171.

Kodama, M., 1993. A new species of highly $CO_2$ tolerant fast growing marine microalga suitable for high-density culture. Journal of Marine Biotechnology, 1, 21-25.

Kuhl A (1974) Phosphorus. In: Stewart WDP (ed) Algal physiology and biochemistry. Blackwell Scientific, Oxford

Kuo, C.T., 2017. Adsorbent-based algal cultivation system to facilitate integration of algal biofuel production with wastewater treatment (Doctoral dissertation, University of Illinois at Urbana-Champaign).

Lakaniemi, A.M., 2012. Microalgal cultivation and utilization in sustainable energy production. Tampereen teknillinen yliopisto. Julkaisu-Tampere University of Technology. Publication; 1034.

Lakaniemi, A.M., Hulatt, C.J., Thomas, D.N., Puhakka, J.A., 2015. Carbon dioxide utilization in gas-sparge microalgal photobioreactors. In Conference paper presented at: Asian biohydrogen, bioproducts symposium. Chongqing, China.

Li, J., Lu, X., 2017. Effect of seasonal temperature on the performance and on the microbial community of a novel AWFR for decentralized domestic wastewater pretreatment. Applied Sciences, 7(6), 605.

Li, X., Xu, H., Wu, Q., 2007. Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnology and Bioengineering, 98(4), 764-771.

Liang, S., Liu, X., Chen, F., Chen, Z., 2004. Current microalgal health food R & D activities in China. In Asian pacific phycology in the 21st century: prospects and challenges (45-48). Springer, Dordrecht.

Lim, S.L., Chu, W.L., Phang, S.M., 2010. Use of *Chlorella vulgaris* for bioremediation of textile wastewater. Bioresource Technology, 101(19), 7314-7322.

Liu, Z.Y., Wang, G.C., Zhou, B.C., 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresource Technology, 99(11), 4717-4722.

Lee, S.H., Oh, H.M., Jo, B.H., Lee, S.A., Shin, S.Y., Kim, H.S., Lee, S.H., Ahn, C.Y., 2014. Higher biomass productivity of microalgae in an attached growth system, using wastewater. Journal of Microbiology and Biotechnology, 24(11), 1566-1573.

Lettinga, G., Rebac, S., Zeeman, G., 2001. Challenge of psychrophilic anaerobic wastewater treatment. Trends in Biotechnology, 19(9), 363-370.

Lorenz, R.T. and Cysewski, G.R., 2000. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. Trends in biotechnology, 18(4), 160-167.

Lundquist, T.J., Woertz, I.C., Quinn, N.W.T., Benemann, J.R., 2010. A realistic technology and engineering assessment of algae biofuel production. Energy Biosciences Institute, 1.

Luo, L., Lin, X., Zeng, F., Luo, S., Chen, Z., Tian, G., 2019. Performance of a novel photobioreactor for nutrient removal from piggery biogas slurry: Operation parameters, microbial diversity and nutrient recovery potential. Bioresource Technology, 272, 421-432.

Lv, J., Guo, J., Feng, J., Liu, Q., Xie, S., 2017. Effect of sulfate ions on growth and pollutants removal of self-flocculating microalga *Chlorococcum* sp. GD in synthetic municipal wastewater. Bioresource Technology, 234, 289-296.

Maestrini, S.Y., Robert, J.M., Leftley, J.W., Collos, Y., 1986. Ammonium thresholds for simultaneous uptake of ammonium and nitrate by oyster-pond algae. Journal of Experimental Marine Biology and Ecology, 102(1), 75-98.

Mane, P.C., Bhosle, A.B., 2012. Bioremoval of Some Metals by Living Algae *Spirogyra* sp. and *Spirullina* sp. from aqueous solution. International Journal of Environmental Research, 6(2), 571-576.

Marjakangas, J.M., Chen, C.Y., Lakaniemi, A.M., Puhakka, J.A., Whang, L.M., Chang, J.S., 2015. Simultaneous nutrient removal and lipid production with *Chlorella vulgaris* on sterilized and non-sterilized anaerobically pretreated piggery wastewater. Biochemical Engineering Journal, 103, 177-184.

Markou, G., Vandamme, D., Muylaert, K., 2014. Using natural zeolite for ammonia sorption from wastewater and as nitrogen releaser for the cultivation of *Arthrospira platensis*. Bioresource Technology, 155, 373-378.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: a review. Renewable and Sustainable Energy Reviews, 14(1), 217-232.

McGinn, P.J., Dickinson, K.E., Park, K.C., Whitney, C.G., MacQuarrie, S.P., Black, F.J., Frigon, J.C., Guiot, S.R., O'Leary, S.J., 2012. Assessment of the bioenergy and bioremediation potentials of the microalga *Scenedesmus* sp. AMDD cultivated in municipal wastewater effluent in batch and continuous mode. Algal Research, 1(2), 155-165.

Mera, R., Torres, E., Abalde, J., 2016. Effects of sodium sulfate on the freshwater microalga *Chlamydomonas moewusii*: implications for the optimization of algal culture media. Journal of Phycology, 52(1), 75-88.

Miron, A.S., Gomez, A.C., Camacho, F.G., Grima, E.M., Chisti, Y., 1999. Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae. Journal of Biotechnology, 70(1-3), 249-270.

Miyachi, S., Kanai, R., Mihara, S., Miyachi, S., Aoki, S., 1964. Metabolic roles of inorganic polyphosphates in *Chlorella* cells. Biochimica et Biophysica Acta (BBA)-General Subjects, 93(3), 625-634.

Mujtaba, G., Choi, W., Lee, C.G., Lee, K., 2012. Lipid production by *Chlorella vulgaris* after a shift from nutrient-rich to nitrogen starvation conditions. Bioresource Technology, 123, 279-283.

Nam, K., Lee, H., Heo, S.W., Chang, Y.K., Han, J.I., 2017. Cultivation of *Chlorella vulgaris* with swine wastewater and potential for algal biodiesel production. Journal of Applied Phycology, 29(3), 1171-1178.

Naylor, A.W., 1970. Phylogenetic aspects of nitrogen metabolism in the algae. Annals of the New York Academy of Sciences, 175(1), 511-523.

Norsker, N.H., Barbosa, M.J., Vermuë, M.H., Wijffels, R.H., 2011. Microalgal production—a close look at the economics. Biotechnology Advances, 29(1), 24-27.

Pahazri, N.F., Mohamed, R.M.S.R., Al-Gheethi, A.A., Kassim, A.H.M., 2016. Production and harvesting of microalgae biomass from wastewater: a critical review. Environmental Technology Reviews, 5(1), 39-56.

Phang, S.M., Miah, M.S., Yeoh, B.G., Hashim, M.A., 2000. *Spirulina* cultivation in digested sago starch factory wastewater. Journal of Applied Phycology, 12(3-5), 395-400.

Polishchuk, A., Valev, D., Tarvainen, M., Mishra, S., Kinnunen, V., Antal, T., Yang, B., Rintala, J., Tyystjärvi, E., 2015. Cultivation of *Nannochloropsis* for eicosapentaenoic acid production in wastewaters of pulp and paper industry. Bioresource Technology, 193, 469-476.

Posadas, E., Szpak, D., Lombó, F., Domínguez, A., Díaz, I., Blanco, S., García-Encina, P.A., Muñoz, R., 2016. Feasibility study of biogas upgrading coupled with nutrient removal from anaerobic effluents using microalgae-based processes. Journal of Applied Phycology, 28(4), 2147-2157.

Pruvost, J., Pottier, L., Legrand, J., 2006. Numerical investigation of hydrodynamic and mixing conditions in a torus photobioreactor. Chemical Engineering Science, 61(14), 4476-4489.

Rawat, I., Kumar, R.R., Mutanda, T., Bux, F., 2011. Dual role of microalgae: phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Applied Energy, 88(10), 3411-3424.

Richmond, A. ed., 2004. Handbook of microalgal culture: biotechnology and applied phycology (Vol. 577). Oxford: Blackwell Science.

Rizwan, M., Mujtaba, G., Memon, S.A., Lee, K., Rashid, N., 2018. Exploring the potential of microalgae for new biotechnology applications and beyond: A review. Renewable and Sustainable Energy Reviews, 92, 394-404.

Roon, R.J., Levenberg, B., 1968. An adenosine triphosphate-dependent, avidin-sensitive enzymatic cleavage of urea in yeast and green algae. Journal of Biological Chemistry, 243(19), 5213-5215.

Sheets, J.P., Yang, L., Ge, X., Wang, Z., Li, Y., 2015. Beyond land application: Emerging technologies for the treatment and reuse of anaerobically digested agricultural and food waste. Waste Management, 44, 94-115.

Shelef, G., Sukenik, A., Green, M., 1984. Microalgae harvesting and processing: a literature review (No. SERI/STR-231-2396). Technion Research and Development Foundation Ltd., Haifa (Israel).

Singh, P., Guldhe, A., Kumari, S., Rawat, I., Bux, F., 2015. Investigation of combined effect of nitrogen, phosphorus and iron on lipid productivity of microalgae *Ankistrodesmus falcatus* KJ671624 using response surface methodology. Biochemical Engineering Journal, 94, 22-29.

Singh, S.P., Singh, P., 2015. Effect of temperature and light on the growth of algae species: a review. Renewable and Sustainable Energy Reviews, 50, 431-444.

Sophonsiri, C., Morgenroth, E., 2004. Chemical composition associated with different particle size fractions in municipal, industrial, and agricultural wastewaters. Chemosphere, 55(5), 691-703.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. Journal of Bioscience and Bioengineering, 101(2), 87-96.

Stockenreiter, M., Haupt, F., Seppälä, J., Tamminen, T., Spilling, K., 2016. Nutrient uptake and lipid yield in diverse microalgal communities grown in wastewater. Algal Research, 15, 77-82.

Tchobanoglous, G., Stensel, H.D., Tsuchinashi, R., Burton, F., Abu-Orf, M., Bowden, G., Pfrang, W., 2014. Wastewater engineering treatment and reuse. New York, US: McGraw-Hill Education.

Thayalakumaran, N., Bhamidimarri, R., Bickers, P.O., 2003. Biological nutrient removal from meat processing wastewater using a sequencing batch reactor. Water Science and Technology, 47(10), 101-108.

Touahria, S., Hazourli, S., Touahria, K., Eulmi, A., Aitbara, A., 2016. Clarification of industrial mining wastewater using electrocoagulation. International Journal of Electrochemical Science, 11(7), 5710-5723.

Umamaheswari, J., Shanthakumar, S., 2016. Efficacy of microalgae for industrial wastewater treatment: a review on operating conditions, treatment efficiency and biomass productivity. Reviews in Environmental Science and Bio/Technology, 15(2), 265-284.

Usha, M.T., Chandra, T.S., Sarada, R., Chauhan, V.S., 2016. Removal of nutrients and organic pollution load from pulp and paper mill effluent by microalgae in outdoor open pond. Bioresource Technology, 214, 856-860.

Van Drecht, G., Bouwman, A.F., Harrison, J., Knoop, J.M., 2009. Global nitrogen and phosphate in urban wastewater for the period 1970 to 2050. Global Biogeochemical Cycles, 23(4).

Viarengo, A, 1985. Biochemical effects of trace metals, Mar. Pollut. Bull. 16,153-158.

Wang, H., Magesan, G.N., Bolan, N.S., 2004. An overview of the environmental effects of land application of farm effluents. New Zealand Journal of Agricultural Research, 47(4), 389-403.

Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y., Ruan, R., 2010. Cultivation of green algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant. Applied Biochemistry and Biotechnology, 162(4), 1174-1186.

Wang, M., Schideman, L., Lu, H., Zhang, Y., Li, B., Cao, W., 2019. Zeolite-amended microalgal-bacterial system in a membrane photobioreactor for promoting system stability, biomass production, and wastewater treatment efficiency to realize Environmental-Enhancing Energy paradigm. Journal of Applied Phycology, 31(1), 335-344.

Wang, Y., Ho, S.H., Cheng, C.L., Guo, W.Q., Nagarajan, D., Ren, N.Q., Lee, D.J., Chang, J.S., 2016. Perspectives on the feasibility of using microalgae for industrial wastewater treatment. Bioresource Technology, 222, 485-497.

Wijffels, R.H., Kruse, O., Hellingwerf, K.J., 2013. Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. Current Opinion in Biotechnology, 24(3), 405-413.

Wright, R.R., Hobbie, J.E., 1966. Use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology, 47(3), 447-464.

Wu, J.Y., Lay, C.H., Chen, C.C., Wu, S.Y., 2017. Lipid accumulating microalgae cultivation in textile wastewater: Environmental parameters optimization. Journal of the Taiwan Institute of Chemical Engineers, 79, 1-6.

Xia, A., Murphy, J.D., 2016. Microalgal cultivation in treating liquid digestate from biogas systems. Trends in Biotechnology, 34(4), 264-275.

Xin, L., Hong-ying, H., Ke, G., Ying-xue, S., 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresource Technology, 101(14), 5494-5500.

Zimmo, O.R., Van der Steen, N.P., Gijzen, H.J., 2003. Comparison of ammonia volatilisation rates in algae and duckweed-based waste stabilisation ponds treating domestic wastewater. Water Research, 37(19), 4587-4594.

Zhang, Z., Huang, J.J., Sun, D., Lee, Y., Chen, F., 2017. Two-step cultivation for production of astaxanthin in *Chlorella zofingiensis* using a patented energy-free rotating floating photobioreactor (RFP). Bioresource Technology, 224, 515-522.

Zhao, L., Zeng, G., Gu, Y., Tang, Z., Wang, G., Tang, T., Shan, Y., Sun, Y., 2019. Nature inspired fractal tree-like photobioreactor via 3D printing for $CO_2$ capture by microaglae. Chemical Engineering Science, 193, 6-14.

Zhu, L., Wang, Z., Shu, Q., Takala, J., Hiltunen, E., Feng, P., Yuan, Z., 2013. Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment. Water Research, 47(13), 4294-4302.

Zhu, C., Zhai, X., Jia, J., Wang, J., Han, D., Li, Y., Tang, Y., Chi, Z., 2018. Seawater desalination concentrate for cultivation of *Dunaliella salina* with floating photobioreactor to produce β-carotene. Algal Research, 35, 319-324.

# 3 Comparison of *Scenedesmus acuminatus* and *Chlorella vulgaris* cultivation in liquid digestates from anaerobic digestion of pulp and paper industry and municipal wastewater treatment sludge


## Abstract

Two microalgae, *Chlorella vulgaris* and *Scenedesmus acuminatus*, were batch cultivated separately in two types of diluted liquid digestates. The first digestate (ADPP) was obtained from a mesophilic laboratory digester treating biosludge from a pulp and paper industry wastewater treatment plant. The second digestate (ADMW) was collected from a full-scale mesophilic anaerobic digester treating mixed municipal wastewater treatment sludge. The highest biomass production (as volatile suspended solids, VSS), 8.2–9.4 g $L^{-1}$, was obtained with *S. acuminatus* in ADPP. *C. vulgaris* in ADMW had the lowest biomass production, reaching 2.0 g $L^{-1}$. Both microalgae removed ammonium efficiently from ADPP (99.9% removal rate) while the final ammonium removal efficiencies from ADMW with *S. acuminatus* and *C. vulgaris* were only 44.0% and 23.8%, respectively. The phosphate removal efficiencies from both ADPP and ADMW were higher than 96.9% with both microalgae. The highest carbohydrate content (60.5%) was obtained with *S. acuminatus* cultivated in ADPP. *S. acuminatus* in ADPP showed one of the highest biomass production yields that has been reported for microalgae in real wastewater-derived nutrient sources. Consequently, this combination is promising for developing biorefinery and biofuel applications in the pulp and paper industry.

## 3.1 Introduction

The pulp and paper industry typically consumes large amounts of wood and water and is among the largest producers of industrial wastewater in the world (Ashrafi et al. 2015). Thus, wastewater treatment is an indispensable part of this industry. However, traditional aerobic wastewater treatment produces vast amounts of biosludge, which is mechanically dewatered as such or mixed with primary sludge and then typically incinerated or landfilled (Stoica et al. 2009). While anaerobic digestion (AD) of the generated biosludge was studied in the 1980s (Puhakka et al. 1988), the recent developments towards biorefineries and circular economy thinking have led to a renewed interest in applying AD for biosludge treatment, as its energy balance is more positive, and it enables simpler nutrient recovery as compared to incineration (Kinnunen et al. 2015). A microalgae-utilising biorefinery concept has been proposed to produce microalgae biomass and to recover nutrients using the liquid effluent of pulp and paper mill-digested residue as a nutrient source for the microalgae (Kinnunen and Rintala 2016; Kouhia et al. 2015). However, pulp and paper mill wastewaters can contain compounds such as lignins, humic acids, furans and dioxins (Ali and Sreekrishnan 2001), which can inhibit microbial growth and, thus, hinder utilisation of the microalgal biomass for products such as biodiesel, biomethane and bioethanol, which require large amount of biomass and cost-efficient cultivation. Microalgal cultivation in pulp and paper mill digestates has been studied previously (Polishchuk et al. 2015; Kinnunen and Rintala 2016) but resulted in low biomass production (0.2 g volatile suspended solids (VSS) per L) (Kinnunen and Rintala 2016).

The cultivation of various microalgal species has been studied using various other waste streams as well (Jia et al. 2016; Molinuevo-Salces et al. 2016; Nam et al. 2016; Posadas et al. 2016). Municipal wastewater is one of the most often used wastewaters due to its large volumes and accessible collection (Tan et al. 2015), and it has been shown to be promising for simultaneous microalgal biomass production and nutrient recovery (Cai et al. 2013a; Tan et al. 2015). In addition to studies on municipal wastewater, microalgae cultivation has also been studied using the liquid fraction of the digestate from AD of municipal wastewater sludge. Tan et al. (2015) succeeded in cultivating *Chlorella pyrenoidosa* outdoors using a diluted liquid fraction of anaerobically digested biosludge, obtaining a maximum biomass concentration of 1.86±0.09 g-VSS $L^{-1}$ during summer, with the photobioreactor temperature ranging from 27.5 to 42.6 °C. This indicates the feasibility of large-scale outdoor microalgal cultures using effluents from AD of sludges as a nutrient source. However, the growth yields

and nutrient recovery efficiency of different microalgal species can be different, even under similar conditions (Abdel-Raouf et al. 2012), which makes it important to find an optimal microalgal species for each application.

The aim of the present study was to assess the feasibility of cultivating microalgal biomass in pulp and paper mill biosludge digestate. Utilising this concept, microalgae cultivation could be integrated in pulp and paper industry biorefinery to produce microalgal biomass (e.g. to biofuel applications while recovering nutrients from the liquid digestate). The digestate from a municipal wastewater treatment plant was used as a reference cultivation medium. The cultivation of two microalgal species, *Chlorella vulgaris* and *Scenedesmus acuminatus*, which were chosen due to their high growth rates and yields as well as their broad use in wastewater treatment studies (Bohutskyi et al. 2015; Wang et al. 2015; Zuliani et al. 2016), was compared in these two digestates.

## 3.2 Materials and methods

### 3.2.1 Microalgal strains and growth medium for seed cultures

*Chlorella vulgaris* (SAG 211-11b) and *Scenedesmus acuminatus* (SAG 38.81) were obtained from the SAG Culture Collection of Algae at the University of Göttingen, Germany as culture suspensions. *C. vulgaris* had been grown in Jaworski's medium (Lakaniemi et al. 2011) and stored frozen at -85 °C for 4 years. After thawing, *C. vulgaris* was inoculated to 100 mL N-8 medium and cultivated in 250 mL Erlenmeyer flasks on an orbital shaker (150 rpm) under fluorescent lamps (Osram L 18W/965 bio lux, Germany) at a light intensity of 40 µmol photons $m^{-2}$ $s^{-1}$ as a seed culture. *S. acuminatus* was inoculated to N-8 medium immediately after obtaining it from the culture collection and cultivated under the same conditions as *C. vulgaris*. The N-8 medium consisted of (g $L^{-1}$): $KNO_3$, 0.5055; $KH_2PO_4$, 0.7400; $Na_2HPO_4$, 0.2598; $MgSO_4 \cdot 7H_2O$, 0.0500; $CaCl_2 \cdot 2H_2O$, 0.0175; $FeNaEDTA \cdot 3H_2O$, 0.0115 and micronutrient ($ZnSO_4 \cdot 7H_2O$, 0.0032; $MnCl_2 \cdot 4H_2O$, 0.013; $CuSO_4 \cdot 5H_2O$, 0.0183; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.0070). The pH of the N-8 medium is naturally 6.5. *C. vulgaris* grew well with that initial pH, whereas there was no growth of *S. acuminatus* in the N-8 medium with an initial pH of 6.5. Based on a previous study by Xu et al. (2015), NaOH was added to adjust the pH to 8.0 for *S. acuminatus* cultivation.

### 3.2.2 Digestates

Digestates from two different sources were studied for microalgal growth. The first digestate (ADPP) was collected from a mesophilic laboratory-scale (6 L) completely stirred tank reactor (hydraulic retention time 14 d and organic loading rate 2.1 kg VS $m^{-3}$ $d^{-1}$) treating biosludge from a pulp and paper industry wastewater treatment plant. The reactor set-ups were as described in Kinnunen et al. (2015), but the biosludge used in this study originated from different pulp and paper mills compared with the data reported in Kinnunen et al. (2015), and thus the digestate characteristics are not directly comparable. The second digestate (ADMW) was collected from a mesophilic anaerobic digester (typically operated at a hydraulic retention time of 20–25 d and organic loading rate of 2.0 kg VS $m^{-3}$ $d^{-1}$) treating mixed sludge in a municipal wastewater treatment plant (Rahola, Tampere, Finland). The digestates were stored at 4 °C until prepared for the cultivation experiments.

To remove particulate solids, both digestates were centrifuged at 5200 rpm for 4 min, and the separated supernatant was filtered through a glass fibre filter (Whatman GF/A, UK) under non-aseptic conditions (not meant to sterilise the wastewater). After filtration, the filtered digestates were stored at 4 °C before use. This study includes two separate cultivation experiments with both digestates. As the filtered digestates were prepared at different times and from different batches of digestates for the two cultivation experiments (Experiments I and II), there were some differences in the digestate compositions (Table 3.1). Considering that the $PO_4^{3-}$-P level may be not sufficient in ADMW, an additional experiment was performed with 0.548 g $L^{-1}$ $K_2HPO_4$ added to the ADMW to enhance microalgal cultivation and nitrogen removal efficiency. Thus, the N/P ratio was adjusted to 7.5, and this ratio was selected as it has been used for high nutrient removal during microalgae cultivation in municipal wastewaters (Cai et al. 2013b; Tan et al. 2015).

**Table 3.1** Characteristics of the filtered digestates originating from the pulp and paper wastewater treatment plant (ADPP) and the municipal wastewater treatment plant (ADMW). Two batches (Experiment I and II) of both filtered digestates were used. The results are presented as the means of n = 2 (2 cultivations, 1 measurements from each); error bars represent standard error

| | ADPP | | ADMW | |
|---|---|---|---|---|
| | Experiment I | Experiment II | Experiment I | Experiment II |
| pH | 8.5 | 8.5 | 8.3 | 8.6 |
| DOC (mg $L^{-1}$) | 370±40 | 210±2 | 530±20 | 560±20 |
| CODs (mg $L^{-1}$) | 910±30 | 900±70 | 1850±40 | 2500±15 |
| TKN (mg $L^{-1}$) | 350±10 | 360±20 | 840±40 | 1000±150 |
| $NH_4^+$-N (mg $L^{-1}$) | 350±50 | 360±1 | 840±130 | 820±10 |
| $NO_3^-$ (mg $L^{-1}$) | <0.5 | <0.5 | <0.5 | <0.5 |
| $NO_2^-$ (mg $L^{-1}$) | <0.5 | <0.5 | <0.5 | <0.5 |
| TP (mg $L^{-1}$) | 28±1 | 20±1 | 10±1 | 14±2 |
| $PO_4^{3-}$-P (mg $L^{-1}$) | 24±1 | 12±0.1 | 2.0±0.2 | 2.5±0.1 |

DOC= dissolved organic carbon

$COD_s$ = soluble chemical oxygen demand

TKN= total Kjeldahl nitrogen

TP= total phosphorus.

### 3.2.3 Microalgal cultivation in digestates

Experiment I was done to select the optimal dilution factor of the liquid digestates for microalgal growth. The digestates were diluted with distilled water, using dilution factors of 5x, 3x and 1.5x and 10x, 7x, 3.5x, 2x and 1x for the ADPP and ADMW, respectively. Using the selected dilution factors, Experiment II was conducted to further study the biomass production, carbon and nutrient removal efficiency and chemical composition of the produced biomass (carbohydrate, lipid and protein). All cultivations were performed in duplicates.

Experiment I was conducted in 1-L photobioreactors, which consisted of a 1-L glass bottle (PYREX) closed with a plastic cap and having two tubes as the gas inlet and outlet. The cultures were bubbled from the bottom with 5% $CO_2$ in the air (v/v) at a flow rate of 0.105 L $min^{-1}$ using a glass distribution tube (porosity 0, ⌀ 22mm, Duran Group, Germany). The photobioreactors were continuously illuminated using white fluorescent lamps (Osram L 18W/965 De Luxe cool daylight, Germany) from two sides of the reactors. It is commonly believed that each microalgal strain has a particular light intensity that is the most optimal for biomass growth (Ho et al. 2012; Xu et al. 2015). Based on preliminary tests (data not shown) in which the microalgae were cultivated separately in N-8 medium at different light intensities, 150 µmol photons $m^{-2}$ $s^{-1}$ and 240 µmol photons $m^{-2}$ $s^{-1}$ were chosen as the light intensities for the cultivation of *C. vulgaris* and *S. acuminatus*, respectively. The inoculum culture was centrifuged to separate cells from the N-8 medium before being mixed with the desired digestate. To identify the accurate microalgal growth medium compositions in Experiment II, samples were taken for analysis of initial dissolved nutrients after inoculation. Each microalgal genus was inoculated to its respective photobioreactor to provide an initial optical density (OD) of 0.20. The initial total culture volume in the reactors was 350 mL for ADPP (the availability was limited) and 700 mL for ADMW. The temperature of the reactors was maintained at 22±2 °C. Distilled water was added to adjust for the water lost through evaporation each time before taking samples for analyses. All cultivations (each combination of different microalgal species with different dilution of digestate) were carried out for 11–12 d.

Experiment II using the selected dilution factors was conducted using similar conditions as Experiment I. The difference was that the initial culture volume in all the cultivations was 700 mL to provide enough volume for the more extensive sampling and more reliable comparison of the growth of the two microalgae in the two digestates. The cultivation duration in Experiment II was 14 d.

### 3.2.4 Analyses and calculations

The culture pH was measured using a WTW 3110 pH meter (WTW, Germany) with a SenTix® 41 electrode (WTW, Germany) in Experiment I and a WTW 330 pH meter (WTW, Germany) with a Slimtrode electrode (Hamilton, Germany) in Experiment II. The light intensity was measured from the outer surface of the photobioreactors by a MQ-200 Quantum Meter (Apogee, USA). The optical density (OD) of the culture samples was measured at a wavelength of 680 nm using a Shimadzu UV-1700 Pharmaspec spectrophotometer after

proper dilution with deionised water to give absorbance values between 0.2–0.7. Light microscopy was carried out using a Zeiss Axioskop 2 equipped with an AxioCam MRc camera. The microalgae cells were first sonicated for 10 min and then observed under the light microscope. Volatile suspended solids (VSS) were measured by filtering 5–15 mL culture solution through a glass fibre filter (Whatman GF/A). Each filter containing the suspended solids was dried at 105 ºC overnight, weighed and then burned in a 550 ºC muffle furnace for 2 h and weighed again. VSS was determined gravimetrically as a difference of the filters after treatment at these two temperatures. The filtrate from VSS filtration was used in the analysis of soluble chemical oxygen demand ($COD_s$), dissolved organic carbon (DOC) and nutrient (N, P) concentration.

$COD_s$ was determined using the dichromate method according to the Finnish Standard SFS 5504. DOC was measured with a total organic carbon analyser (Shimadzu Model TOC-5000) with an ASI-5000 autosampler. Total nitrogen was measured as total Kjeldahl nitrogen (TKN) with the Tecator Kjeltec Systems (FOSS Tecator Digestor 8 and KT 200 Kjeltec, Sweden), and total phosphorus (TP) was measured with a Hach kit LCK349 (0.05–1.5 mg $L^{-1}$ $PO_4$-P) or LCK350 (2.0–20.0 mg $L^{-1}$ $PO_4$-P), according to the manufacturer's instructions. $NH_4^+$-N was measured with an ion selective electrode (Thermo Scientific Orion ISE meter). The ammonium removal rate was calculated as ARR=($C_0$-$C_t$) $t^{-1}$, where $C_0$ is the ammonium concentration on day 0, and $C_t$ is the ammonium concentration when the ammonium concentration had fallen below 0.5 mg $L^{-1}$, which indicated >99% $NH_4^+$-N removal. The possible significance of ammonium stripping was estimated by calculating the fraction of unionised ammonium with the following equation (Emerson et al. 1975) as rate of ammonia stripping has been shown to correlate well with free ammonia concentration (Zimmo et al. 2003):

$$unionised\ NH_3(\%) = \frac{100}{1 + 10^{(pK_a - pH)}}\,, \tag{3.1}$$

where $pK_a = 0.09018 + \frac{2729.92}{T}$ and $\mathrm{T} = \mathrm{temperature(ºK)}$.

$NO_3^-$, $NO_2^-$ and $PO_4^{3-}$ were measured using an ICS-1600 ion chromatograph (Dionex, USA) with an AS-DV autosampler, Ion- Pac AS4A-SC anion exchange column and ASRS-300 suppressor (2 mm). The eluent contained 1.9 mM $Na_2CO_3$ and 1.7 mM $NaHCO_3$, and the eluent flow rate was 1 mL $min^{-1}$.

The composition of the produced microalgal biomass (proteins, carbohydrates, and lipids) was measured from the freeze-dried biomass. Before freeze-drying, the algal culture was centrifuged at 5200 rpm for 2 min, and the supernatant was discarded. The harvested microalgae samples were dried in a vacuum freeze dryer (Christ ALPHA 1-4 LD plus) for 24 h. The protein content of the produced biomass was measured with a protein assay kit, based on the method of Bradford (Bio-Rad Protein Assay Dye Reagent Concentration; Protein Standard II). The total carbohydrate concentration of the algal biomass was measured with the anthrone method after hot alkaline extraction (Chen and Vaidyanathan 2013). In short, 10 mg dried *microalgal* pellets were resuspended in 0.2 mL distilled water and then heated in 0.4 mL 40% (w/v) KOH at 90 °C for 1 h. After cooling down, the sample was mixed with 1.2 mL cold absolute ethanol and stored in a fridge at −20 °C overnight. The pellet was resuspended in 1.5 mL distilled water after discarding the supernatant. An aliquot (0.2 mL) of the sample was mixed and vortexed with 0.4 mL of pre-chilled 75% $H_2SO_4$ solution (stored at 4 °C) in a test tube. To this, 0.8 mL of the anthrone reagent (2 g $L^{-1}$ in 75% $H_2SO_4$, freshly prepared) was added, and then the mixture was subsequently boiled at 100 °C for 15 min. After cooling, the absorbance was read at 578 nm using a Shimadzu UV-1700 Pharmaspec spectrophotometer. The blank absorbance of the sample was read by reacting 0.2 mL of the sample with 1.2 mL 75% $H_2SO_4$ without the anthrone reagent. The amount of carbohydrate was estimated using a standard curve created using d-glucose. The total lipid content of the biomass was measured by extracting the lipids with chloroform/methanol and determining the lipids gravimetrically. An aliquot (50 mg) of freeze-dried microalgal biomass was mixed with 10 mL of chloroform/methanol (2/1, v/v) and then sonicated for 5 min. After sonication, the mixture was reacted for 4 h on a magnetic stirrer at 1000 rpm. Then, 5 mL of distilled water were added to the mixture and centrifuged together at 3000 rpm for 2–3 min. Lipids remained in the chloroform after centrifugation, and then the chloroform (8 mL) was placed in a pre-weighted tube. The nitrogen was sparged to remove chloroform for 2 h and lipid content was left in the tube; the tube was then weighed again.

## 3.3 Results

### 3.3.1 Selection of the dilution factor for the digestates

The growth of *Chlorella vulgaris* and *Scenedesmus acuminatus* was tested with different dilutions with the pulp and paper mill digestate (ADPP; 5x, 3x and 1.5x) and municipal sludge

digestate (ADMW; 10x, 7x, 5x, 3.5x, 2x and 1x) to study the growth of the two microalgae in the two digestates at similar initial ammonium concentrations. As shown in Table 3.2, both *C. vulgaris* and *S. acuminatus* had the highest biomass production in 2x diluted ADMW and 1.5x diluted ADPP. Compared with the growth of both microalgae in ADMW, the biomass production in ADPP was much higher (maximum VSS=9.4±0.8 g $L^{-1}$ of *S. acuminatus* and VSS=5.1±0.6 g $L^{-1}$ of *C. vulgaris*). In fact, the obtained biomass production was among the highest reported for microalgal cultivations that have been conducted in real wastewater (Table 3.3). The biomass production of both microalgae was lower in undiluted ADMW, and it is likely that microalgal growth was limited by the higher ammonium concentration (840 mg $L^{-1}$) and brownish colour of the undiluted digestate. The initial ammonium concentrations in 2x diluted ADMW and 1.5x diluted ADPP were 420 mg $L^{-1}$ and 230 mg $L^{-1}$, respectively, whereas the corresponding phosphate concentrations were 1.0 mg $L^{-1}$ and 16.0 mg $L^{-1}$, respectively. As the biomass production was the highest at these conditions, 2x diluted ADMW and 1.5x diluted ADPP were selected for the more detailed study of biomass production, nutrient removal and algal biomass composition in Experiment II.

**Table 3.2** Ammonium-N and phosphate-P concentrations and biomass production of *Chlorella vulgaris* and *Scenedesmus acuminatus* cultivated in diluted digestates from a pulp and paper mill wastewater treatment plant (ADPP) and a municipal wastewater treatment plant (ADMW). The results of biomass production as the means of n = 4 (2 cultivations, 2 measurements from each); error bars represent standard deviation. The results of ammonium-N and phosphate-P are presented as the means of n = 2 (2 cultivations, 1 measurements from each); error bars represent standard error.

| | ADPP | | | ADMW | | | | |
|---|---|---|---|---|---|---|---|---|
| Dilution factor | 5x | 3x | 1.5x | 10x | 7x | 3.5x | 2x | 1x |
| Ammonium-N (mg $L^{-1}$) | 70±10 | 115±15 | 230±35 | 84±10 | 120±20 | 240±40 | 420±65 | 840±130 |
| Phosphate-P (mg $L^{-1}$) | 4.8±0.2 | 8±0.3 | 16±0.7 | 0.20±0.0 | 0.29±0.0 | 0.57±0.1 | 1.0±0.1 | 2.0±0.2 |
| *C. vulgaris* VSS (g $L^{-1}$) | 1.9±0.2 | 3.0±0.1 | 5.1±0.9 | 0.6±0.1 | 0.6±0.2 | 1.1±0.1 | 1.2±0.1 | 0.9±0.1 |
| *S. acuminatus* VSS (g $L^{-1}$) | 6.1±3.1 | 6.2±2.3 | 9.4±1.1 | 0.8±0.1 | 0.9±0.1 | 1.7±0.1 | 2.2±0.1 | 2.1±0.2 |

**Table 3.3** Maximum biomass concentrations and chemical compositions of the produced biomass from selected studies in which microalgae have been cultivated in real wastewaters and synthetic media. The digestates were from the pulp and paper wastewater treatment plant (ADPP; 1.5x diluted) and the municipal wastewater treatment plant (ADMW; 2x diluted)

| Medium | Microalgae | Maximum biomass concentration (g $L^{-1}$) | Carbohydrates (%) | Lipids (%) | Proteins (%) | Reference |
|---|---|---|---|---|---|---|
| ADPP | *Chlorella vulgaris* | 2.91[a)] | 6.8 | 21.7 | 30.3 | This study |
| ADPP | *Scenedesmus acuminatus* | 8.22 [a)] | 60.5 | 19.9 | 24.3 | This study |
| ADMW | *Chlorella vulgaris* | 2.02 [a)] | 6.3 | 23.0 | 41.8 | This study |
| ADMW | *Scenedesmus acuminatus* | 2.92 [a)] | 44.3 | 35.9 | 28.0 | This study |
| Anaerobic digested poultry litter | *Scenedesmus bijuga* | 0.38 | 22.9 | 9.5 | 39.0 | Singh et al. (2011) |
| Human urine | *Chlorella sorokiniana* | 9.3 | n.a.[b)] | n.a. | n.a. | Tuantet et al. (2014) |
| Anaerobic digested municipal wastewater | *Chlorella pyrenoidosa* | 1.97 | 13.9 | 10.9 | 60.7 | Tan et al. (2015) |
| Anaerobic treated piggery wastewater | *Chlorella vulgaris* | 3.24 | n.a. | 32 | n.a. | Marjakangas et al. (2015) |
| Swine manure | *Chlorella vulgaris, Scenedesmus obliquus, Chlamydomonas reindhardtii* | 1.25 | 50 | 20 | 25 | Molinuevo-Salces et al. (2016) |
| Anaerobic digested sewage | Scenedesmus sp. and/or *Chlorella* sp. | 0.42 | n.a. | n.a. | n.a. | Viruela et al. (2016) |
| Tris–acetate–phosphate medium | *Chlamydomonas reinhardtii* UTEX 90 | 12.4 | 59.7 | n.a. | 9.2 | Choi et al. (2010) |
| Modified Basal Medium | *Chlorella vulgaris* FSP-E | 7.22 | 50.4 | n.a. | n.a. | Ho et al. (2013) |

Note: This table gives an indication of the range of microalgal biomass production and cell compositions obtained in various studies but the given values cannot be explicitly compared as the studies have been conducted using different growth conditions (photobioreactor design, light intensity, $CO_2$ addition, nutrient concentration etc.).
[a)] Only biomass values from Experiment II are reported, as biomass composition was not measured in Experiment I
[b)] n.a.=data not available

### 3.3.2 Algal growth and nutrient removal efficiency

In Experiment II, the microalgal growth was studied in more detail using the selected dilutions with both ADPP and ADMW. Of the two different digestates, both microalgae grew better in ADPP when compared to ADMW and reached their highest biomass concentrations (*C. vulgaris*: 2.9 g $L^{-1}$; *S. acuminatus*: 8.2 g $L^{-1}$) on day 14 (Figure 3.1A). In ADMW, *S. acuminatus* reached a maximum biomass concentration of 2.9 g $L^{-1}$ and *C. vulgaris* of 2.0 g $L^{-1}$. The biomass concentration of *S. acuminatus* in ADPP was higher than that detected for the other cultivations from day 2 onwards. On day 7, the biomass concentration of *S. acuminatus* in ADPP was already 4.9 g $L^{-1}$, while in other cultivations biomass concentrations remained below 3.0 g $L^{-1}$ on day 14.

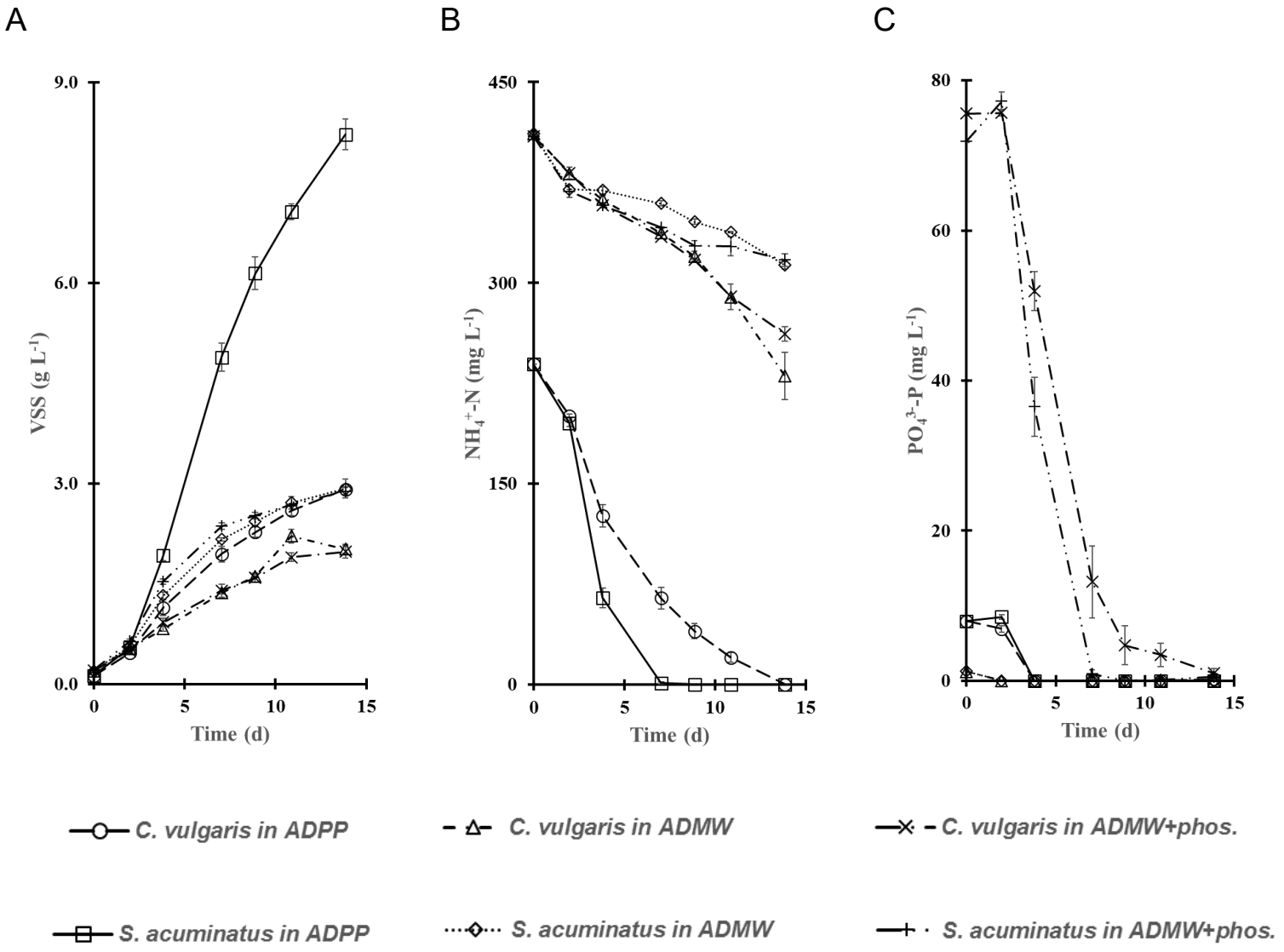



**Figure 3.1** Microalgal biomass concentration (as g VSS $L^{-1}$) (A), the soluble ammonium-N (B) and phosphate-P concentrations (C) during the cultivation of *Chlorella vulgaris* and *Scenedesmus acuminatus* in the digestates from the pulp and paper wastewater treatment plant (ADPP; 1.5x diluted), the municipal wastewater treatment plant (ADMW; 2x diluted) and the municipal wastewater treatment plant supplied with phosphorus (ADMW+phos.; 2x diluted). The results of VSS and phosphate-P are presented as the means of n = 4 (2 cultivations, 2 measurements from each); error bars represent standard deviation. The results of ammonium-N are presented as the means of n = 2 (2 cultivations, 1 measurements from each); error bars represent standard error.

Both microalgae were able to remove ammonium efficiently from ADPP, in which the ammonium concentration decreased from 240 mg $L^{-1}$ to 0.1 mg $L^{-1}$ during cultivation of both microalgal species, resulting in a 99.9% removal efficiency (Figure 3.1B). Interestingly, the same amount of ammonium and phosphorus was removed by both algae in ADPP, even though the biomass production for *S. acuminatus* (8.2 g $L^{-1}$) was more than two times higher than that for *C. vulgaris* (2.9 g $L^{-1}$). The ammonium was, however, removed faster by *S. acuminatus* (26.5 mg $L^{-1}$ $d^{-1}$) than by *C. vulgaris* (17.1 mg $L^{-1}$ $d^{-1}$). From ADMW, which had an initial ammonium concentration of 410 mg $L^{-1}$, the ammonium removal efficiencies were much lower, being only 44.0% and 23.8% with *S. acuminatus* and *C. vulgaris*, respectively (Figure 3.1B).

The initial phosphate concentration in ADPP was 8.0 mg $L^{-1}$ while in ADMW it was much lower (1.3 mg $L^{-1}$, Figure 3.1C), apparently due to phosphorus removal using chemical precipitation in the municipal wastewater treatment plant. The phosphate levels in ADPP and ADMW decreased rapidly to below the detection limit of 0.1 mg $L^{-1}$ by both microalgae, in 4 days with ADPP and 2 days with ADMW. Thus, the phosphate removal efficiencies were higher than 96.9% in all four cultivations (Figure 3.1C). An additional experiment performed to assess the effects of phosphate addition to the ADMW (initial phosphate concentration was 73.8±1.8 mg $L^{-1}$, added as $K_2HPO_4$) resulted in 99% removal of phosphate within 9 days with *S. acuminatus* and 14 days with *C. vulgaris* but similar biomass production and ammonium removal efficiency as cultivations without extra phosphate (Figure 3.1).

### 3.3.3 COD and DOC during microalgal cultivation in the digestates

It is essential to measure COD in wastewater treatment, as it is a typical indicator of the water quality. The initial $COD_s$ value in the 2x diluted ADMW was 1259±5 mg $L^{-1}$, which was approximately two times the initial value present in the 1.5x diluted ADPP having $COD_s$ of 600±34 mg $L^{-1}$. In ADPP, the $COD_s$ removal efficiencies of *C. vulgaris* and *S. acuminatus* were 27.6% and 36.1%, respectively (Figure 3.2A). In ADMW, the highest $COD_s$ removal efficiency was obtained with *C. vulgaris* (55.4%), while *S. acuminatus* was able to remove 48.7% of the initial $COD_s$. DOC is a typical parameter measured from microalgal cultivations, as DOC is usually released during microalgal photosynthesis and can support bacterial growth (Watanabe et al. 2005; Hulatt and Thomas 2010). The DOC concentration in ADPP was stable and remained close to the initial value during the whole cultivation period (Figure 3.2B). A similar amount of DOC was removed from ADMW by the two microalgae (26.0% by *C. vulgaris*, 24.8% by *S. acuminatus*) (Figure 3.2B).

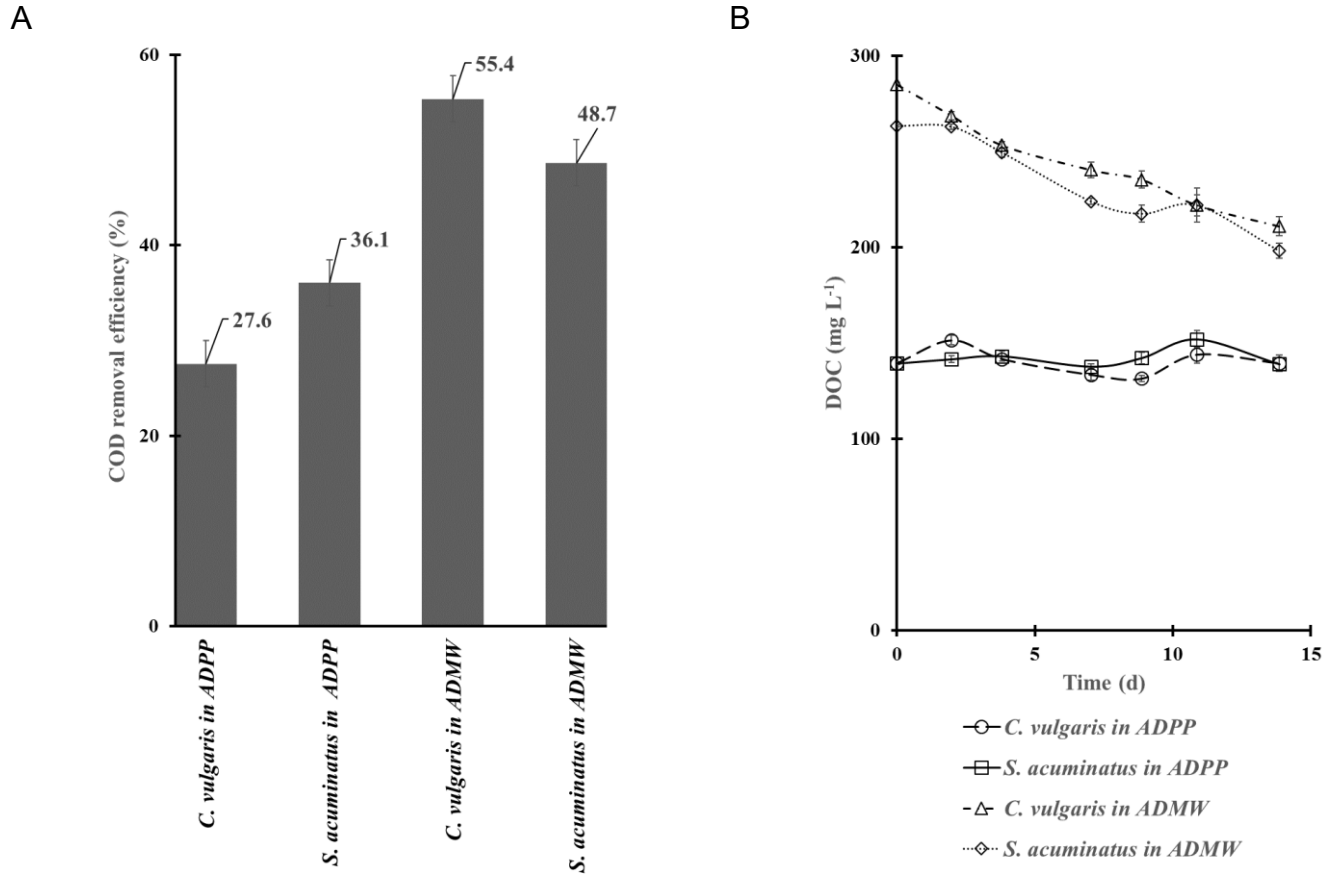



**Figure 3.2** COD removal efficiency (A) and DOC concentration (B) during the cultivation of *Chlorella vulgaris* and *Scenedesmus acuminatus* in the digestates from the pulp and paper wastewater treatment plant (ADPP; 1.5x diluted) and the municipal wastewater treatment plant (ADMW; 2x diluted). The results are presented as the means of n = 4 (2 cultivations, 2 measurements from each); error bars represent standard deviation.

### 3.3.4 Chemical composition and morphological changes of the microalgae

Among all studied cultures, *S. acuminatus* in ADPP had the highest carbohydrate content (60.5%) per dry weight, whereas a carbohydrate content of only 6.8% was measured from the dried cells of *C. vulgaris* cultivated in ADPP (Table 3.3). Similarly, the carbohydrate content of *S. acuminatus* and *C. vulgaris* grown in ADMW were 44.3% and 6.3%, respectively.

*C. vulgaris* is spherical in shape while *S. acuminatus* is spindle-shaped (Figure 3.3). No morphological differences in the *C. vulgaris* cells in the two digestates were observed between day 4 and day 14 (these cultivation days represent nitrogen-sufficient and nitrogen-limited conditions in ADPP). The cell size (diameter) of *C. vulgaris* was about 5–10 µm in both studied digestates during the whole cultivation period. However, clear morphological changes of *S. acuminatus* were detected in both digestates between day 4 and day 14. In ADPP, the cell length of *S. acuminatus* increased from 20 to 22.5 µm on overage while the width increased from 6.25 to 7.5 µm on average. In ADMW, the cell length of the *S. acuminatus* decreased from an average of 30 to 25 µm while the width increased from an average of 8.75 to 11.25 µm. Slightly different types of changes in cell morphology were observed in a previous study, in which the cell length size of *Scenedesmus* sp. was found to increase from 4.5 to 5.3 µm while the cell width size decreased from 3.36 to 2.44 µm when cultivated under a nitrate-limited condition (Pancha et al. 2014). Thus, there was no clear correlation between nitrogen availability and the cell size.

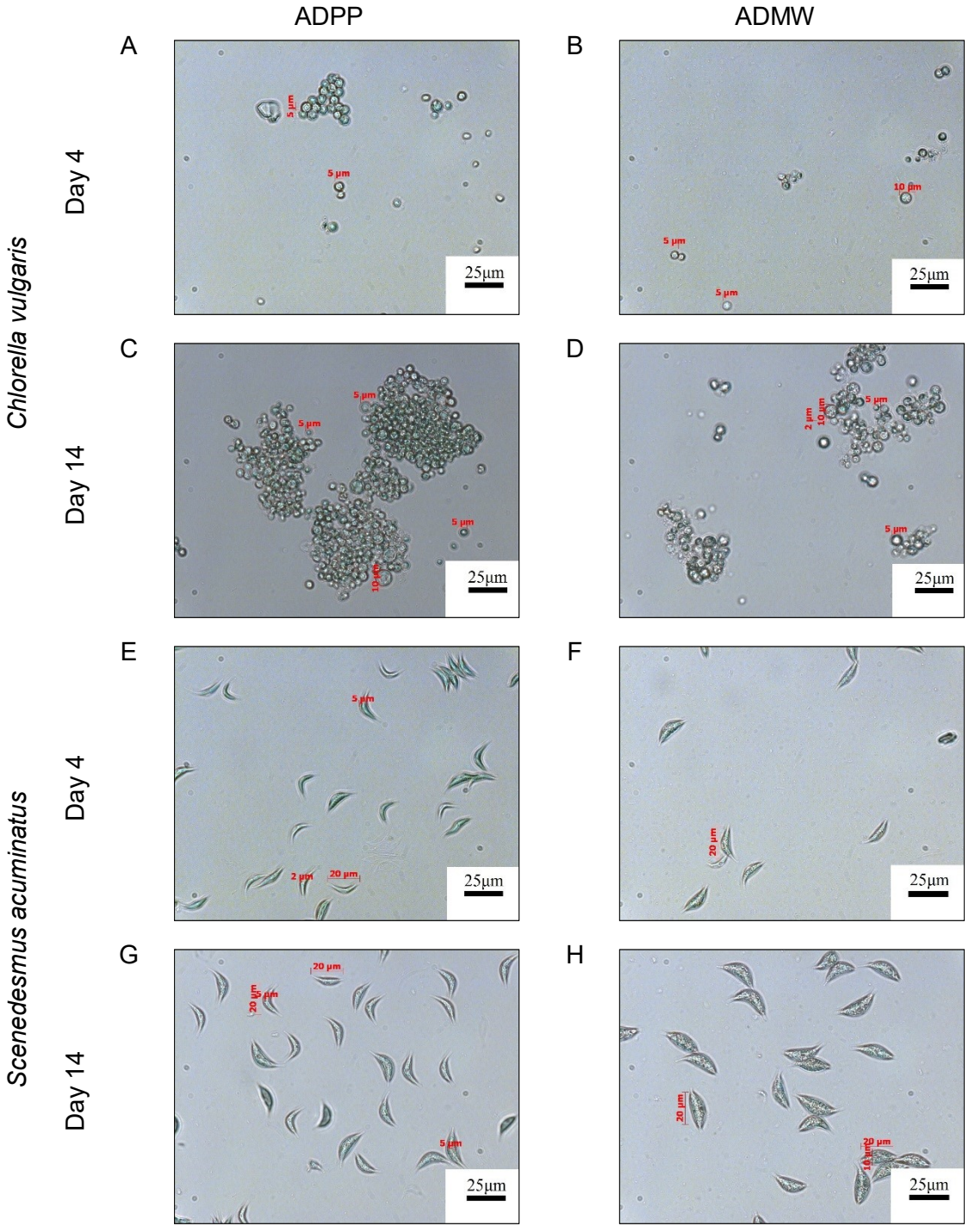



**Figure 3.3** Microscope photos of the microalgal cells: *Chlorella vulgaris* in ADPP (A)(C); *Chlorella vulgaris* in ADMW (B), (D); *Scenedesmus acuminatus* in ADPP (E), (G) and *Scenedesmus acuminatus* in ADMW (F), (H) on day 4 and day 14, respectively. Digestates were from the pulp and paper wastewater treatment plant (ADPP; 1.5x diluted) and the municipal wastewater treatment plant (ADMW; 2x diluted)

## 3.4 Discussion

This study was carried out in batch to select microalgal species that enable high biomass production and efficient nutrient removal from pulp and paper mill biosludge digestate and to assess the potential of pulp and paper mill biosludge digestate as a cultivation medium compared to the more commonly used municipal wastewater treatment digestate. The biomass production of *S. acuminatus* cultivated in ADPP (8.2–9.4 g $L^{-1}$) in this study was among the highest obtained when microalgae have been cultivated in real wastewater, while several studies have reported high microalgal biomass production (7.22–12.4 g $L^{-1}$) in artificial growth medium (Table 3.3).

The selection of medium dilution plays an important role in microalgal cultivation since the dilution will change the medium turbidity (thus light penetration) and nutrient concentrations (Posadas et al. 2016; Wang et al. 2010; Xia and Murphy 2016). High ammonia concentrations have been shown to inhibit microalgal growth, whereas too low nutrient concentrations can limit growth (Britto and Kronzucker 2002; Tan et al. 2015). In contrast to our study, Franchino et al. (2013) chose higher dilution ratios (1:10, 1:15, 1:20 and 1:25) as optimum to ensure the microalgal growth due to the high digestate medium turbidity. However, higher dilutions reduced the concentrations of nutrients, which could result in lower microalgal biomass production (Franchino et al. 2013; Wang et al. 2010). Instead of clean water, Bohutskyi et al. (2016) mixed 1–20% anaerobic digestion centrate (ADC) with primary and secondary wastewater effluents separately to cultivate several types of microalgal strains, and they found that 5–10% ADC succeeded in improving microalgal growth and productivity in both effluents due to the additional nutrients and optimum nitrogen-to-phosphorus ratio.

The present study shows a high microalgal biomass yield is possible in the liquid digestates from pulp and paper wastewater treatment plant biosludge. The growth of *S. acuminatus* appeared to be similar level in both ADPP (8.2–9.4 g-VSS $L^{-1}$) and ADMW (2.2–2.9 g-VSS $L^{-1}$) in Experiment I and II, while *C. vulgaris* growth differed more between the two experiments, with both digestates being higher in ADPP in Experiment I (5.1 vs. 2.9 g-VSS $L^{-1}$) and in ADMW in Experiment II (2.0 vs. 1.2 g-VSS $L^{-1}$). Even though a strict comparison between the two cultivations is not justified due to different sampling dates and slightly different cultivation conditions, this shows the repeatability of the high biomass production of *S. acuminatus* in ADPP. On the other hand, the growth of *C. vulgaris* appeared to be more sensitive to cultivation conditions even when including the differences in the compositions

of the digestates in Experiments I and II (Table 3.1). Similarly, in the previous study, the growth of *C. vulgaris* has been found to vary (0.31–0.19 g-VSS $L^{-1}$) when using even synthetic growth medium (Kinnunen and Rintala 2016).

Several possible reasons (e.g. algal species, medium characteristics and microbial community) could explain the different growth yields in the cultivations of this study. Kinnunen and Rintala (2016) obtained a concentration of 0.17 g $L^{-1}$ (VSS) when *Scenedesmus sp*. was cultivated in a liquid digestate from a different pulp and paper mill. The growth of this different *Scenedesmus* species was much lower than the biomass production obtained with *S. acuminatus* in ADPP in this study. Lignin, which ends up in pulp and paper mill wastewaters, is an amorphous polymer that is difficult for microorganisms to degrade (Higuchi 1990). In addition, some of the polyphenolic compounds in softwood knots, such as pinosylvins, have antimicrobial activity (Välimaa et al. 2007), while lignin and its derivatives are quite toxic to certain microorganisms, such as microalgae and cyanobacteria (Ball et al. 2001). It has been reported that *S. subspicatus* was much more resistant than *C. vulgaris* and *Microcystis aeruginosa* to the chemicals released from barley straw (e.g. 2 phenyl-phenol, p-cresol and benzaldehyde) (Murray et al. 2010). This indicates that *C. vulgaris* was more susceptible to the chemical compounds likely present in ADPP, which may have caused the much lower biomass production obtained with *C. vulgaris* than with *S. acuminatus* in ADPP. When microalgae are cultivated in wastewaters or digestates, microbes are always present and might affect the growth of microalgae. In the present study, the indigenous microbial communities of the two digestates (ADPP and ADMW) were likely different since they originated from different types of sources and had very different chemical compositions. Studies have shown that certain bacteria can enhance bacterial growth, whereas certain bacteria can inhibit it (Croft et al. 2005; Santos and Reis 2014). For example, De-Bashan et al. (2004) reported that *Azospirillum brasilense* strain Cd stimulated the growth of *C. vulgaris* and *C. sorokiniana* when they were co-immobilised in small alginate beads. Interestingly, a similar genus, *Azospirillum lipoferum*, was found in an aerated plug-flow lagoon that was used to treat pulp and paper mill effluent (Yu and Mohn 2001). However, De Bashan et al. (2004) did not study the effect of *Azospirillum brasilense* on *S. acuminatus*, and therefore it is not possible to compare the effect of *Azospirillum* to the growth of *C. vulgaris* and *S. acuminatus*. Lee et al. (2016) assumed that the reason for the slow growth of *S. quadricauda* in municipal wastewater might be related to *Alcaligenes*, which was an abundant bacterium in the wastewater. Some species of *Alcaligenes* genus have been shown to cause cell lysis and the death of certain cyanobacteria (Manage et al., 2000), and others have been shown to

have nitrification and denitrification abilities that may affect ammonium removal and nitrogen availability to the microalgae (Joo et al. 2005). The interactions between bacteria and microalgae have been shown to be very species specific, even in the same medium (Schäfer et al. 2002). In our study, certain a bacterium present in the studied ADPP may have enhanced the growth of *S. acuminatus* but not the growth of *C. vulgaris.* Alternatively, a certain bacterium could have inhibited *C. vulgaris* but not *S. acuminatus*.

The present results demonstrate efficient nutrient (ammonium and phosphorus) removal by both microalgae from ADPP, while different nutrient removal efficiencies were obtained in ADMW with the two different microalgal strains. Beuckels et al. (2015) reported that *C. vulgaris* was able to accumulate more nitrogen into biomass than *S. obliquus*. This likely happened in this study with ADMW, as the decrease in $NH_4^+$-N concentration was higher with *C. vulgaris* than with *S. acuminatus* (Figure 3.1B), although the biomass growth of *C. vulgaris* was somewhat lower (Figure 3.1A). Several possible ammonium transformations (algal uptake, ammonia stripping, bacterial growth and nitrification) can happen in algae–bacteria consortium systems, such as microalgal cultures in unsterilised wastewater (Bohutskyi et al. 2015; González-Fernández et al. 2011; He et al. 2013; Zimmo et al. 2003). In this study, the nitrate and nitrite levels in both liquid digestates were low (<1.0 mg $L^{-1}$) during the whole cultivation. This means the possibility of ammonium removal by nitrification was small. As the pH varied in all cultures between 7.5 and 8.0 and the average temperature was 22°C, the theoretical fraction of unionised ammonia in all cultivations was 1.4%–4.4%. This suggests that some stripping of the unionised ammonia may have occurred but that the main portion of the removed ammonium from the digestates was used for microbial growth. The removed phosphorus could be taken up into the microalgal cells as polyphosphates and/or cell components or precipitate from the medium due to high pH (Cai et al. 2013a, b). Thus, it seems that the higher initial phosphate concentration of ADPP was not the reason for the higher biomass production observed in ADPP than in ADMW.

While there was no big difference in DOC removal with *C. vulgaris* and *S. acuminatus* when the same digestate was used, the difference in DOC trends between ADPP and ADMW emphasise their differences as a cultivation medium. One reason for stable DOC in ADPP could be that the released DOC from photosynthetic microalgal cells equalled to the consumed DOC for growth of hetertrophic organisms (such as bacteria). Decrease in $COD_s$ suggests, however, that higher level of organic compounds was degraded during the cultivation than was released as DOC by the microalage. $COD_s$ was not fully removed during

the cultivations, indicating that treatments other than biological methods could be required for further $COD_s$ removal after microalgal harvesting.

The nutrient and carbon removal levels from ADPP were similar with both *C. vulgaris* and *S. acuminatus*, but the biomass production of *S. acuminatus* was much higher than that of *C. vulgaris*. Based on the typical biochemical composition of microalgae, it is estimated that about 50% of the microalgal biomass is carbon (Chisti 2008). Thus, 1.0–4.1 g $L^{-1}$ carbon was required to produce the microalgal biomass, as the obtained VSS values ranged between 2.0 and 8.2 g $L^{-1}$ for the two microalgae (Figure 3.1A). However, the total removed dissolved carbon from the digestates was below 150 mg $L^{-1}$. Hence, $CO_2$ supply contributed to the microalgal growth as the main carbon source, indicating that most of the microalgal biomass was produced via photoautotrophic growth.

Based on the chemical formulas of the main components of microalgae (carbohydrate: $C_6H_{10}O_5$, lipid: $C_{57}H_{104}O_6$ and protein: $C_{1.9}H_{3.8}ON_{0.5}P_{0.031}$) (Kouhia et al. 2015), nitrogen only appears in proteins. It is assumed that microalgae using the same amount of nitrogen should produce the same amount of protein. However, in this study, despite the similar ammonium removal, the protein content of *C. vulgaris* was 6–13.8 percentage units higher than that of *S. acuminatus* in the same digestate, whereas *S. acuminatus* contained significantly more carbohydrates and produced more biomass than *C. vulgaris*. Nitrogen deficiency can cause a reduction in protein content (Diniz et al. 2016) along with an enhancement of energy-rich products, such as carbohydrates and lipids (de Farias Silva and Bertucco 2015; Siaut et al. 2011). In this study, the produced microalgal biomass likely contained mainly proteins at the beginning due to the sufficient nitrogen in the cultures, and the microalgal carbon was allocated to energy-rich compounds after the ammonium was consumed completely. Similarly, when microalga *Chlamydomonas reinhardtii* was exposed to environmental stress such as nitrogen starvation, starch accumulation was first observed and reached high levels by day 2 (approximately 60 µg per million cell), and after extended nitrogen limitation (5 days), oil accumulation reached a maximal level (40 µg per million cell) (Siaut et al. 2011). The carbohydrate and lipid contents of *C. vulgaris* in ADMW and ADPP were in a similar range, while the protein content of *C. vulgaris* in ADMW was higher than that in ADPP (Table 3.3), likely due to the higher initial nitrogen concentration of ADMW compared to that of ADPP. However, as the sum of the analysed biochemical components (58.8%–71.1%) from *C. vulgaris* was much lower than 100%, it is not certain whether carbohydrate or lipid accumulation occurred in *C. vulgaris*. The sum of proteins, lipids and carbohydrates in *C. vulgaris* has also been reported to be lower than 70% in previous studies (Lakaniemi et al. 2011; Sydney et

al. 2010). Burczyk et al. (2014) suggested that low levels of polyamines (PAs) in the cell walls of microalgae might enhance the action of lytic enzymes, and they found that the PA content in *C. vulgaris* strain 140 was 4 to 5 times higher than that in *S. obliquus* strain 633. Thus, it is possible that in this study and also in the previous studies reporting sums of proteins, carbohydrates (sugars) and lipids to be clearly below 100%, the high PA content in *C. vulgaris* may have hindered the cell lysis during the analysis of the biochemical components. In addition, carbohydrates might have been lost due to the alkali dissolution during the measurement (Kane and Roth 1974).

## 3.5 Conclusions

*Chlorella vulgaris* and *Scenedesmus acuminatus* were shown to be able to grow and remove nutrients in liquid digestates from both a pulp and paper industry wastewater treatment plant (ADPP) and a municipal wastewater treatment plant (ADMW). *S. acuminatus* in 1.5-times diluted ADPP enabled the highest biomass production of 8.2–9.4 g $L^{-1}$, which is among the highest yields reported for microalgae cultivated in wastewaters. The maximum biomass yield was also much higher than the growth of *C. vulgaris* in 1.5-times diluted ADPP (2.9 g $L^{-1}$) as well as the growth of *S. acuminatus* (2.9 g $L^{-1}$) and *C. vulgaris* (2.0 g $L^{-1}$) in 2-times diluted ADMW. Phosphate and ammonium removal efficiencies were high with both microalgae from ADPP (over 97%). Both algae were able to remove phosphate from ADMW, although the ammonium removal efficiencies remained low (24–44%). According to the results obtained in this study, cultivation of *S. acuminatus* in pulp and paper mill biosludge digestates is a promising approach for producing a carbohydrate-rich biomass with a high yield and cheap nutrient supply (e.g. for biogas and bioethanol production). Future studies on semi-continuous or continuous cultivation systems and biomass harvesting could further promote the practical applications.

## References


Abdel-Raouf N, Al-Homaidan AA, Ibraheem IBM (2012) Microalgae and wastewater treatment. Saudi J Biol Sci 19:257-275.

Ali M, Sreekrishnan TR (2001) Aquatic toxicity from pulp and paper mill effluents: a review. Adv Environ Res 5:175-196.

Ashrafi O, Yerushalmi L, Haghighat F (2015) Wastewater treatment in the pulp-and-paper industry: A review of treatment processes and the associated greenhouse gas emission. J Environ Manage 158:146-157.

Ball AS, Williams M, Vincent D, Robinson J (2001) Algal growth control by a barley straw extract. Bioresour Technol 77:177-181.

Beuckels A, Smolders E, Muylaert K (2015) Nitrogen availability influences phosphorus removal in microalgae-based wastewater treatment. Water Res 77:98-106.

Bohutskyi P, Kligerman DC, Byers N, Nasr LK, Cua C, Chow S, Su C, Tang Y, Betenbaugh MJ, Bouwer EJ (2016) Effects of inoculum size, light intensity, and dose of anaerobic digestion centrate on growth and productivity of *Chlorella* and *Scenedesmus* microalgae and their poly-culture in primary and secondary wastewater. Algal Res 19:278-290.

Bohutskyi P, Liu K, Nasr LK, Byers N, Rosenberg JN, Oyler GA, Betenbaugh MJ, Bouwer EJ (2015) Bioprospecting of microalgae for integrated biomass production and phytoremediation of unsterilized wastewater and anaerobic digestion centrate. Appl Microbiol Biotechnol 99:6139-6154.

Britto DT, Kronzucker HJ (2002) $NH_4^+$ toxicity in higher plants: a critical review. J Plant Physiol 159:567-584.

Burczyk J, Zych M, Ioannidis NE, Kotzabasis K (2014) Polyamines in cell walls of *Chlorococcalean* microalgae. Z NATURFORSCH C 69:75-80.

Cai T, Park SY, Li Y (2013a) Nutrient recovery from wastewater streams by microalgae: status and prospects. Renew Sustainable Energy Rev 19:360-369.

Cai T, Park SY, Racharaks R, Li Y (2013b) Cultivation of *Nannochloropsis salina* using anaerobic digestion effluent as a nutrient source for biofuel production. Appl Energy 108:486-492.

Chen Y, Vaidyanathan S (2013) Simultaneous assay of pigments, carbohydrates, proteins and lipids in microalgae. Anal Chim Acta 776:31-40.

Chisti Y (2008) Biodiesel from microalgae beats bioethanol. Trends Biotechnol 26:126-131.

Choi SP, Nguyen MT, Sim SJ (2010) Enzymatic pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production. Bioresour Technol 101:5330-5336.

Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin $B_{12}$ through a symbiotic relationship with bacteria. Nature 438:90-93.

De-Bashan LE, Hernandez JP, 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.

de Farias Silva CE, Bertucco A (2016) Bioethanol from microalgae and cyanobacteria: A review and technological outlook. Process Biochem 51:1833-1842.

Diniz GS, Silva AF, Araújo OQF, Chaloub RM (2016) The potential of microalgal biomass production for biotechnological purposes using wastewater resources. J Appl Phycol 29:821-832.

Emerson K, Russo RC, Lund RE, Thurston RV (1975) Aqueous ammonia equilibrium calculations: effect of pH and temperature. J Fish Res Board Can 32:2379-2383.

Franchino M, Comino E, Bona F, Riggio VA (2013) Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate. Chemosphere 92:738-744.

González-Fernández C, Molinuevo-Salces B, García-González MC (2011) Nitrogen transformations under different conditions in open ponds by means of microalgae–bacteria consortium treating pig slurry. Bioresour Technol 102:960-966.

He PJ, Mao B, Lü F, Shao LM, Lee DJ, Chang JS (2013) The combined effect of bacteria and *Chlorella vulgaris* on the treatment of municipal wastewaters. Bioresour technol 146:562-568.

Higuchi T (1990) Lignin biochemistry: biosynthesis and biodegradation. Wood Sci Technol 24:23-63.

Ho SH, Chen CY, Chang JS (2012) Effect of light intensity and nitrogen starvation on $CO_2$ fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresour Technol 113:244-252.

Ho SH, Huang SW, Chen CY, Hasunuma T, Kondo A, Chang JS (2013) Characterization and optimization of carbohydrate production from an indigenous microalga *Chlorella vulgaris* FSP-E. Bioresour Technol 135:157-165.

Hulatt CJ, Thomas DN (2010) Dissolved organic matter (DOM) in microalgal photobioreactors: a potential loss in solar energy conversion?. Bioresour Technol 101:8690-8697.

Jia Q, Xiang W, Yang F, Hu Q, Tang M, Chen C, Wang G, Dai S, Wu H, Wu H (2016) Low-cost cultivation of *Scenedesmus* sp. with filtered anaerobically digested piggery wastewater: biofuel production and pollutant remediation. J Appl Phycol 28:727-736.

Joo HS, Hirai M, Shoda M (2005) Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* No. 4. J Biosci Bioeng 100:184-191.

Kane SM, Roth R (1974) Carbohydrate metabolism during ascospore development in yeast. J Bacteriol 118:8-14.

Kinnunen V, Rintala J (2016) The effect of low-temperature pretreatment on the solubilization and biomethane potential of microalgae biomass grown in synthetic and wastewater media. Bioresour Technol 221:78-84.

Kinnunen V, Ylä-Outinen A, Rintala J (2015) Mesophilic anaerobic digestion of pulp and paper industry biosludge–long-term reactor performance and effects of thermal pretreatment. Water Res 87:105-111.

Kouhia M, Holmberg H, Ahtila P (2015) Microalgae-utilizing biorefinery concept for pulp and paper industry: Converting secondary streams into value-added products. Algal Res 10:41-47.

Lakaniemi AM, Hulatt CJ, Thomas DN, Tuovinen OH, Puhakka JA (2011) Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass. Biotechnol Biofuels 4:34.

Lee J, Lee J, Shukla SK, Park J, Lee TK (2016) Effect of algal inoculation on COD and nitrogen removal, and indigenous bacterial dynamics in municipal wastewater. J Microbiol Biotechnol 26:900-908.

Manage PM, Kawabata Z, Nakano SI (2000) Algicidal effect of the bacterium *Alcaligenes denitrificans* on *Microcystis* spp. Aquat Microb Ecol 22:111-117.

Marjakangas JM, Chen CY, Lakaniemi AM, Puhakka JA, Whang LM, Chang JS (2015) Simultaneous nutrient removal and lipid production with *Chlorella vulgaris* on sterilized and non-sterilized anaerobically pretreated piggery wastewater. Biochem Eng J 103:177-184.

Molinuevo-Salces B, Mahdy A, Ballesteros M, González-Fernández C (2016) From piggery wastewater nutrients to biogas: microalgae biomass revalorization through anaerobic digestion. Renew Energy 96:1103-1110.

Murray D, Jefferson B, Jarvis P, Parsons SA (2010) Inhibition of three algae species using chemicals released from barley straw. Environ Technol. 31:455-466.

Nam K, Lee H, Heo SW, Chang YK, Han JI (2016) Cultivation of *Chlorella vulgaris* with

swine wastewater and potential for algal biodiesel production. J Appl Phycol:1-8.

Pancha I, Chokshi K, George B, Ghosh T, Paliwal C, Maurya R, Mishra S (2014) Nitrogen stress triggered biochemical and morphological changes in the microalgae *Scenedesmus* sp. CCNM 1077. Bioresour Technol 156:146-154.

Pettersen RC (1984) The chemistry of Solid Wood. American Chemical Society 207:57-126.

Polishchuk A, Valev D, Tarvainen M, Mishra S, Kinnunen V, Antal T, Yang B, Rintala JA, Tyystjärvi E (2015) Cultivation of *Nannochloropsis* for eicosapentaenoic acid production in wastewaters of pulp and paper industry. Bioresour Technol 193:469-476.

Posadas E, Szpak D, Lombó F, Domínguez A, Díaz I, Blanco S, García-Encina PA, Muñoz R (2016) Feasibility study of biogas upgrading coupled with nutrient removal from anaerobic effluents using microalgae-based processes. J Appl Phycol 28:2147-2157.

Puhakka JA, Viitasaari MA, Latola PK, Määttä RK (1988) Effect of temperature on anaerobic digestion of pulp and paper industry wastewater sludges. Water Sci Technol 20:193-201.

Santos CA, Reis A (2014) Microalgal symbiosis in biotechnology. Appl Microbiol Biotechnol 98:5839-5846.

Schäfer H, Abbas B, Witte H, Muyzer G (2002) Genetic diversity of 'satellite'bacteria present in cultures of marine diatoms. FEMS Microbiol Ecol 42:25-35.

Siaut M, Cuiné S, Cagnon C, Fessler B, Nguyen M, Carrier P, Beyly A, Beisson F, Triantaphylidès C, Li-Beisson Y, Peltier G (2011) Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves. BMC Biotechnol 11:7.

Singh M, Reynolds DL, Das KC (2011) Microalgal system for treatment of effluent from poultry litter anaerobic digestion. Bioresour Technol 102:10841-10848.

Stoica A, Sandberg M, Holby O (2009) Energy use and recovery strategies within wastewater treatment and sludge handling at pulp and paper mills. Bioresour Technol 100:3497-3505.

Sydney EB, Sturm W, de Carvalho JC, Thomaz-Soccol V, Larroche C, Pandey A, Soccol CR (2010) Potential carbon dioxide fixation by industrially important microalgae. Bioresour Technol 101:5892-5896.

Tan XB, Yang LB, Zhang YL, Zhao FC, Chu HQ, Guo J (2015) *Chlorella pyrenoidosa* cultivation in outdoors using the diluted anaerobically digested activated sludge. Bioresour Technol 198:340-350.

Tuantet K, Temmink H, Zeeman G, Janssen M, Wijffels RH, Buisman CJ (2014) Nutrient

removal and microalgal biomass production on urine in a short light-path photobioreactor. Water Res 55:162-174.

Välimaa AL, Honkalampi-Hämäläinen U, Pietarinen S, Willför S, Holmbom B, von Wright A (2007) Antimicrobial and cytotoxic knotwood extracts and related pure compounds and their effects on food-associated microorganisms. Int J Food Microbiol 115:235-243.

Viruela A, Murgui M, Gómez-Gil T, Durán F, Robles Á, Ruano MV, Ferrer J, Seco A (2016) Water resource recovery by means of microalgae cultivation in outdoor photobioreactors using the effluent from an anaerobic membrane bioreactor fed with pre-treated sewage. Bioresour Technol 218:447-454.

Wang L, Li Y, Chen P, Min M, Chen Y, Zhu J, Ruan RR (2010) Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. Bioresour Technol 101:2623-2628.

Wang Z, Zhao Y, Ge Z, Zhang H, Sun S (2015) Selection of microalgae for simultaneous biogas upgrading and biogas slurry nutrient reduction under various photoperiods. J Chem Technol Biotechnol 91:1982-1989.

Watanabe K, Takihana N, Aoyagi H, Hanada S, Watanabe Y, Ohmura N, Saiki H, Tanaka H (2005) Symbiotic association in *Chlorella* culture. FEMS Microbiol Ecol 51:187-196.

Xia A, Murphy JD (2016) Microalgal cultivation in treating liquid digestate from biogas systems. Trends Biotechnol 34:264-275.

Xu X, Shen Y, Chen J (2015) Cultivation of *Scenedesmus dimorphus* for C/N/P removal and lipid production. Electron J Biotechn 18:46-50.

Yu Z, Mohn WW (2001) Bacterial diversity and community structure in an aerated lagoon revealed by ribosomal intergenic spacer analyses and 16S ribosomal DNA sequencing. Appl Environ Microbiol 67:1565-1574.

Zimmo OR, Van der Steen NP, Gijzen HJ (2003) Comparison of ammonia volatilisation rates in algae and duckweed-based waste stabilisation ponds treating domestic wastewater. Water Res 37:4587-4594.

Zuliani L, Frison N, Jelic A, Fatone F, Bolzonella D, Ballottari M (2016) Microalgae cultivation on anaerobic digestate of municipal wastewater, sewage sludge and agro-waste. Int J Mol Sci 17:1692.

# 4 Cultivation of *Scenedesmus acuminatus* in different liquid digestates from anaerobic digestion of pulp and paper industry biosludge


## Abstract

Different undiluted liquid digestates from mesophilic and thermophilic anaerobic digesters of pulp and paper industry biosludge with and without thermal pretreatment were characterized and utilized for cultivating *Scenedesmus acuminatus*. Higher *S. acuminatus* biomass yields were obtained in thermophilic digestates (without and with pretreatment prior to anaerobic digestion (AD): 10.2±2.2 and 10.8±1.2 g $L^{-1}$, respectively) than in pretreated mesophilic digestates (7.8±0.3 g $L^{-1}$), likely due to differences in concentration of sulfate, iron, and/or other minor nutrients. *S. acuminatus* removed over 97.4% of ammonium and 99.9% of phosphate and sulfate from the digestates. Color (74–80%) and soluble COD (29–39%) of the digestates were partially removed. Different AD processes resulted in different methane yields (18–126 L $CH_4$ $kg^{-1}$ VS), digestate compositions, and microalgal yields. These findings emphasize the importance of optimizing each processing step in wood-based biorefineries and provide information for pulp and paper industry development for enhancing value generation.

## 4.1 Introduction

Due to environmental pollution and climate change, the European Union has promoted a binding goal of reducing greenhouse gas emissions by at least 40% in each member country by 2030 compared to 1990, including a 27% share of renewable energy for the EU (European Council, 2014). With the rapid growth of and heavy dependence on fossil fuels in Asia (Lee et al., 2017) as well as in other regions (e.g., North America, Latin America, and Africa) (Tan et al., 2017), a series of policies and legislations to encourage a low-carbon economy and green growth should be implemented. Biomass, which refers to all organic material originating from plants (e.g., algae, trees, and crops), can be converted into biofuels and energy carriers and is therefore a major renewable energy feedstock (McKendry, 2002). Compared with terrestrial plants, microalgae have great potential as a sustainable bioenergy feedstock due to, e.g., higher growth rates, no requirements for arable land, and the potential of wastewater treatment to recover nutrients (Guldhe et al., 2017).

However, before microalgae can be commercially utilized in low-value products such as energy and fuels (Arenas et al., 2017), higher biomass yields need to be generated to make the process more economically feasible. Since wastewater can provide the water and nutrients for the microalgae, many studies have been carried out to cultivate microalgae in different kinds of wastewaters, including municipal, agricultural, and industrial wastewater (Lv et al., 2017; Guldhe et al., 2017; Kinnunen and Rintala, 2016). Microalgal cultivation in anaerobic digestion (AD) effluents, as a specific waste stream, has shown significant potential for biorefinery applications due to efficient nutrient removal and accumulation of high-value products (e.g., astaxanthin, carotenoids, and omega-3 fatty acids) in microalgal biomass (Polishchuk et al., 2015; Xia and Murphy, 2016). The integration of AD effluents from pulp and paper industry biosludge and microalgal cultivation (hereafter referred to as integrated AD&MC system) has been studied to produce biomass and recover nutrients from wastewater (Kinnunen and Rintala, 2016; Polishchuk et al., 2015). The results of our previous study (Tao et al., 2017) indicated the possibility of high-yield microalgal biomass production and efficient nutrient removal when *Scenedesmus acuminatus* was cultivated in liquid digestates from the AD of pulp and paper industry biosludge.

The pulp and paper industry is a water- and energy-intensive biomass-refining industry that typically treats its wastewaters in aerobic systems, which generate a large amount of primary sludge and biosludge. The AD of the generated sludge has gained increasing attention

within the pulp and paper industry due to, e.g., methane production as a renewable energy (Kinnunen et al., 2015; Veluchamy and Kalamdhad, 2017) and the possibility for nutrient recovery. Thermal pretreatment prior to AD is one of the main approaches used to enhance methane production from pulp and paper industry biosludge (Kamali et al., 2016; Kinnunen et al., 2015). To understand the effect of thermal pretreatment temperatures (80 °C, 105 °C, 121 °C, and 134 °C) on the potential for methane production from biosludge in the pulp and paper industry, Kinnunen et al. (2015) carried out methane potential batch assays at 35 °C. They reported that methane production was increased by 39–140% compared to untreated biosludge with increasing pretreatment temperatures, except for methane production from biosludge treated at the lowest temperature, 80 °C, which was lower than that obtained from untreated biosludge. However, although increased pretreatment temperatures increased methane production, costs and energy consumption increased as well (Kinnunen et al., 2015). To our knowledge, the first full-scale AD plant integrated with a pulp mill for digesting pulp mill sludge is currently being planned in Finland (Liikanen, 2016).

Previous studies have shown that biosludge with different treatments (pretreatment and AD) can result in different methane production yields and digestate compositions (Asunis, 2015; Kinnunen et al., 2015). To optimize an integrated AD&MC system for maximum bioenergy (methane and microalgal biomass) production, it is important to study each component and thus provide an overview of the AD&MC system itself. The aim of this work was to study *S. acuminatus* cultivation in various types of liquid digestates from the AD of pulp and paper industry biosludge, which to our knowledge has not been studied before. The objective was to provide scientifically and practically relevant information to pulp and paper industry biorefineries that consider implementing AD of biosludge and microalgal cultivation in the resulting liquid digestate. The following research questions were addressed: (1) How do different AD conditions change the composition of the digestates and in turn affect the growth of *S. acuminatus*? (2) Can *S. acuminatus* grow in and simultaneously remove nutrients from undiluted digestates from pulp and paper mill biosludge? The microalga *S. acuminatus* was chosen due to its high growth rate and ability to grow in various types of waste streams (Adamsson, 2000; Tao et al., 2017).

## 4.2 Materials and Methods

### 4.2.1 Microalgal strain and liquid digestates

*Scenedesmus acuminatus* (SAG 38.81) was obtained as a culture suspension from the SAG Culture Collection of Algae at the University of Göttingen, Germany. The stock culture was maintained in 100 mL of modified N-8 medium (Praveenkumar et al., 2014) in a 250-mL Erlenmeyer flask on an orbital shaker (150 rpm) and continuously illuminated using fluorescent lamps (Osram L 18W/965 Biolux, Germany) at a light intensity of 40 µmol photos $m^{-2}$ $s^{-1}$. Since there was no growth of *S. acuminatus* in the modified N-8 medium with an initial pH of 6.5, the pH was adjusted to 8.0 by adding 5 M NaOH. Based on a previous study by Xu et al. (2015), 8.0 is an optimal initial pH for the cultivation of *Scenedesmus* sp.

Four types of digestates characterized in this study were collected from anaerobic, semi-continuously fed, completely stirred tank reactors (5 L liquid volume) treating biosludge from a pulp and paper industry wastewater treatment plant (Asunis, 2015). Three different pulp and paper mill biosludge digestates used in the microalgal cultivation experiments of the present study were anaerobically digested at 55 °C (thermophilic digestate, T), anaerobically digested at 55 °C after thermal pretreatment at 121 °C for 10 min (pre-treated thermophilic digestate, Tp), and anaerobically digested at 35 °C after thermal pretreatment at 121 °C for 10 min (pre-treated mesophilic digestate, Mp). The fourth pulp and paper mill biosludge digestate referred to in this paper was anaerobically digested at 35 °C (mesophilic digestate, M) (Asunis, 2015) and utilized for the cultivation of *S. acuminatus* in our previous study (Tao et al., 2017). The digestates were centrifuged at 5200 rpm for 4 min, and the supernatant was filtered through a glass fiber filter (Whatman GF/A, UK). After filtration, the liquid digestates (Fig. S1 in the Supplementary Material) were stored at 4 °C before being used.

The microalgal growth results with the mesophilic digestate (M) are not directly comparable to the three digestates used for microalgal cultivation in the present study because, in our previous study, *S. acuminatus* was grown in 1.5-times diluted mesophilic digestate M (Tao et al., 2017), whereas in this study *S. acuminatus* was cultivated in undiluted digestates. Therefore, growth yields of *S. acuminatus* in digestate M were not compared to the microalgal cultivation results obtained in this study. However, the composition of the digestate M was provided in order to show more clearly how the digestate characteristics change depending on the AD temperature and presence or absence of a pretreatment step.

### 4.2.2 Photobioreactors

*S. acuminatus* was grown separately in the three different digestates (digestate refers to liquid, filtered digestate) for 21 days in photobioreactors (four replicates with each digestate), which consisted of a 1-L glass bottle (Pyrex) sealed with a plastic cap, with two tubes penetrating the cap serving as the gas inlet and outlet. Air with 5% $CO_2$ (v/v) at a flow rate of 0.105 L $min^{-1}$ was sparged from the bottom by a glass distribution tube (porosity 0, ⌀ 22 mm, Duran Group, Germany). The photobioreactors were continuously illuminated using white fluorescent lamps (Osram L 18W/965 De Luxe Cool Daylight, Germany) with a light intensity of 240 µmol photos $m^{-2}$ $s^{-1}$ (Xu et al., 2015) from two sides of the reactors. *S. acuminatus* was inoculated to the photobioreactors to provide an initial optical density ($OD_{680}$) of 0.2. The initial total culture volume in the reactors was 600 mL. The temperature of the reactors was maintained at 22±2 °C. Water evaporated during the cultivation due to the constant sparging, and therefore distilled water was added to compensate for the evaporated water volume (marked with lines on the photobioreactors) each time before taking samples for analyses.

### 4.2.3 Analytical methods

The culture pH was measured using a WTW 330 pH meter (WTW, Germany) with a Slimtrode electrode (Hamilton, Germany). The light intensity was controlled by measuring the average value of six sites on two sides of the photobioreactors' outer surface by a MQ-200 quantum meter (Apogee, USA).

Volatile suspended solids (VSS) were measured by filtering 10–15 mL of culture solution through a glass fiber filter (Whatman GF/A) to assess microalgal biomass production. Each filter containing the suspended solids was dried at 105 ºC overnight, then weighed and burned in a 550 ºC muffle furnace for 2 h before being weighed again. VSS was determined gravimetrically as the difference between the filters after treatment at these two temperatures. The supernatant after VSS filtration was used in the analysis of digestate color ($ODd_{680}$) and turbidity, soluble chemical oxygen demand (soluble COD), soluble biochemical oxygen demand ($BOD_7$s), dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), and nutrient (N, P, S) concentrations. The OD was measured at a wavelength of 680 nm using a Shimadzu UV-1700 Pharmaspec spectrophotometer after proper dilution with distilled water to give absorbance values between 0.2–0.7. Turbidity was measured with a TN-100/T-

100 turbidimeter. $OD_{680}$ was also measured from non-filtrated samples to assess microalgal biomass production ($ODm_{680}$).

The growth rates were calculated using the following equation:

$$\mu = \frac{\ln(X_t/X_0)}{t - t_0} \quad (4.1)$$

where $X_0$ is the concentration of biomass measured as VSS (g $L^{-1}$) at initial time ($t_0$) and $X_t$ is the concentration of biomass at a specific time (t).

Soluble COD was determined using a dichromate method according to the Finnish Standard SFS 5504. The determination of $BOD_7$s was achieved with a WTW OxiTop Control/OxiTop measuring system. DOC and DIC were measured with a total organic carbon analyzer (Shimadzu Model TOC-5000) with an ASI-5000 autosampler. $NH_4^+$-N was measured with an ion-selective electrode (Thermo Scientific Orion ISE meter). The nutrients' (ammonium, phosphate, and sulfate) removal rate was calculated as NRR = $(C_0 - C_t)\ t^{-1}$, where $C_0$ is the nutrient concentration on day 0, and $C_t$ is the nutrient concentration after decreasing to below 0.1 mg $L^{-1}$, which represents > 99.9% nutrient removal. $NO_3^-$, $NO_2^-$, $PO_4^{3-}$, and $SO_4^{2-}$ were measured using an ICS-1600 ion chromatograph (Dionex, USA) with an AS-DV autosampler, Ion-Pac AS4A-SC anion exchange column, and ASRS-300 suppressor (2 mm). The system was operated in isocratic mode using an eluent containing 1.9 mM $Na_2CO_3$ and 1.7 mM $NaHCO_3$, and an eluent flow rate of 1 mL $min^{-1}$.

## 4.3 Results and Discussion

### 4.3.1 Characteristics of the liquid digestates

The four pulp and paper industry biosludge digestates originating from digesters operating at different temperatures to treat biosludge with and without thermal pretreatment had different characteristics (Table 4.1). The initial pH of all the digestates was above 8.0, and the buffering capacity was good because the pH remained relatively stable in all cultivations despite efficient ammonium utilization, the uptake of which usually decreases culture pH, as shown by, e.g., Goldman and Brewer (1980). The $ODd_{680}$ of the thermophilic digestates were higher than those of the mesophilic digestates. In addition, the $ODd_{680}$ of the digestates

indicated that pretreatment leads to increased color, as their $ODd_{680}$ were slightly higher than those without pretreatment. Digestate Tp showed the darkest color ($ODd_{680}$: 0.63±0.08; turbidity: 320 NTU) of all the digestates. However, the $ODd_{680}$ of digestate T (0.59±0.06) was higher than that of Mp (0.35±0.01), while its turbidity (280 NTU) was lower than that of Mp (290 NTU). The correlation between $ODd_{680}$ and turbidity is unclear, likely due to the different wavelengths used in the two measurements. Substances in the liquid digestates responsible for their color may include clay, silt, finely divided inorganic and organic matter, soluble-colored organic compounds, plankton, and other microscopic organisms (Wang et al., 2010). The turbidity of liquid digestates may vary, ranging from, e.g., 2960 to 51400 NTU in the liquid fraction of mainly manure digestates from 11 full-scale co-digestion plants (Akhiar et al., 2017). The turbidities of our samples were much lower than those in Akhiar et al. (2017), likely due to different sampling methods. In the study of Akhiar et al. (2017) the liquid fractions of the digestates were separated from the solids either by screw press, centrifugation or vibrating screen, whereas in this study digestates were centrifuged and then filtered through glass fiber filters with a nominal pore size of 1.6 µm. The dark color of the medium, which results in poor light penetration, is one of the issues that could reduce microalgal growth (Wang et al., 2010; Xia and Murphy, 2016). For example, in a study by Wang et al. (2010) where *Chlorella* sp. were cultivated in a liquid fraction (filtered through glass microfiber filters with pore size of 1.5 µm) of anaerobically digested dairy manure (turbidity: 1800–1900 NTU) with different dilutions (10-, 15-, 20-, and 25-times) for 21 days, the inverse correlation between turbidity and specific algal growth rates ($R^2$ = 0.982) indicated that high turbidity may limit algal growth. However, dilution for the benefit of microalgal growth increases total wastewater treatment volume and might actually reduce microalgal growth due to a reduction in nutrients and trace element concentrations.

**Table 4.1** Composition of the liquid digestates from the anaerobic digestion of the pulp and paper industry biosludge produced under thermophilic conditions without pretreatment (T) and with pretreatment (121 °C) for 10 min (Tp) and under mesophilic conditions without pretreatment (M) and with pretreatment (121 °C) for 10 min (Mp).

| | T | Tp | M [a)] | Mp |
|---|---|---|---|---|
| pH | 8.2 | 8.3 | 8.5 | 8.3 |
| Alkalinity (mg $L^{-1}$ $CaCO_3$) | 2700 | 3100 | n.a.[b)] | 2600 |
| $ODd_{680}$ | 0.59 ± 0.06 | 0.63 ± 0.08 | 0.34 ± 0.01 | 0.35 ± 0.01 |
| Turbidity (NTU) | 280 | 320 | n.a. | 290 |
| $NH_4^+$-N (mg $L^{-1}$) | 380 ± 20 | 480 ± 20 | 350 ± 50 | 380 ± 15 |
| $NO_3^-$ (mg $L^{-1}$) | <1.0 | <1.0 | <1.0 | <1.0 |
| $NO_2^-$ (mg $L^{-1}$) | <1.0 | <1.0 | <1.0 | <1.0 |
| TP [a)] (mg $L^{-1}$) | 33 ± 3 | 27 ± 1 | 28 ± 1 | 33 ± 2 |
| $PO_4^{3-}$-P (mg $L^{-1}$) | 16 ± 3 | 15 ± 3 | 18 ± 1 | 15 ± 1 |
| N:P[c)] | 12.1 ± 2.3 | 17.6 ± 1.5 | 12.5 ± 2.0 | 11.6 ± 1.0 |
| $SO_4^{2-}$-S[a)] (mg $L^{-1}$) | 17 ± 1.0 | 15 ± 0.1 | 17 ± 0.9 | 3 ± 0.1 |
| Soluble COD (mg $L^{-1}$) | 1200 ± 130 | 2000 ± 130 | 910 ± 30 | 1170 ± 10 |
| $BOD_7s$ [a)] (mg $L^{-1}$) | 110 ± 5 | 60 ± 100 | n.a. | 60 ± 5 |
| $BOD_7s$/soluble COD [a)] | 0.09 ± 0.04 | 0.03 ± 0.77 | n.a. | 0.05 ± 0.50 |
| DOC (mg $L^{-1}$) | 300 ± 4 | 540 ± 110 | 370 ± 40 | 150 ± 0 |
| DIC (mg $L^{-1}$) | 570 ± 10 | 690 ± 46 | 520 ± 5 | 680 ± 47 [a)] |

a) The values with ± sign include standard errors (*n* = 2)

b) n.a. = data not available

c) N:P (mass per mass): N refers to $NH_4^+$-N and P refers to TP

The thermophilic digestates (T and Tp) had on average 65 mg $L^{-1}$ higher ammonium concentrations compared with the mesophilic digestates (M and Mp). In addition, the pretreatment also led to increased ammonium concentration in the digestate especially in the case of thermophilic digestion. The digestate Tp had on average 100 mg $L^{-1}$ higher ammonium concentration than digestate T (Table 4.1). Ammonium was available in all the digestates as a nitrogen source for microalgal growth, while nitrate and nitrite concentrations were below 1.0 mg $L^{-1}$. The sulfate-S concentration in digestate Mp was much lower than corresponding concentrations in the other three digestates (Table 4.1). The total phosphorus content was similar (27–30 mg $L^{-1}$) in all the digestates, and approximately 50% of the phosphorus existed in the form of phosphate — except in digestate M, where the phosphate share was slightly higher (64.3%). Xin et al. (2010) have reported an optimal N/P ratio (mass per mass) for *Scenedesmus* sp. LX1 growth to range between 5 and 8, while *Scenedesmus* sp. in the study of Rhee (1978) required an N/P ratio of approximately 13.5 to grow without limitations by either nutrient. The optimal ratio is also species-specific. The N/P ratios of the digestates in this study ranged from 12 to 18 (Table 4.1) and were thus somewhat higher than the reported values. However, no extra phosphate was added to the digestates since it did not help with microalgal biomass production or ammonium removal in the digestates of sewage sludge in our previous study (Tao et al., 2017).

A phenomenon similar to that with ammonium was observed with soluble COD values of the different digestates. The thermophilic digestates had higher soluble COD values than the mesophilic digestates; and when the digestates produced at the same digestion temperature were compared, those generated with pretreatment resulted in higher soluble COD values than those without pretreatment (Table 4.1). The $BOD_7$s/soluble COD ratios were lower than 1:20 in the measured digestates (T, Tp, and Mp), which means that most of the organic material left in the liquid digestates after anaerobic digestion was not easily biodegradable. The DIC concentration (520–690 mg $L^{-1}$) of each digestate was higher than the corresponding DOC concentration (150–540 mg $L^{-1}$).

### 4.3.2 Cultivation of *S. acuminatus* in the liquid digestates

#### 4.3.2.1 Microalgal biomass production

Microalgal biomass production as indicated by VSS in the three studied digestates (T, Tp, and Mp) was as shown in Figure 4.1. The $ODm_{680}$ and VSS had a positive correlate in each digestate (T: $R^2 = 0.96$; Tp: $R^2 = 0.96$; Mp: $R^2 = 0.97$). The final microalgal biomass concentration after 21 days of batch cultivation was higher with both thermophilic digestates (T, Tp: 10.2±2.2–10.8±1.2 g $L^{-1}$) than the concentration obtained with the mesophilic digestate (Mp: 7.8±0.3 g $L^{-1}$). Despite the relatively high initial ammonium concentrations (380–480 mg $L^{-1}$) in all cultures, no clear lag phase was observed in microalgal growth. The biomass concentration started to stabilize on day 15–18. *S. acuminatus* in digestate Tp initially grew more slowly than in digestates T and Mp, likely due to its higher initial ammonium concentration potentially inhibiting or slowing down photosynthesis (Abeliovich and Azov, 1976) as well as poorer light penetration (due to the darker color of the digestate). Before day 9, the *S. acuminatus* biomass concentration in digestate T (6.0 g-VSS $L^{-1}$ at day 9) was the highest, followed by *S. acuminatus* in digestate Mp (4.9 g-VSS $L^{-1}$ at day 9) and Tp (4.4 g-VSS $L^{-1}$ at day 9). After day 9 and day 15, the VSS concentration in digestate Tp exceeded that in digestates Mp and T, respectively. The highest specific growth rates for all digestates were obtained during different periods (Table 4.2). These values are relatively high, as previous studies have reported growth rates ranging from 0.41 to 1.06 $day^{-1}$ (Diniz et al., 2017; Wang et al., 2010).

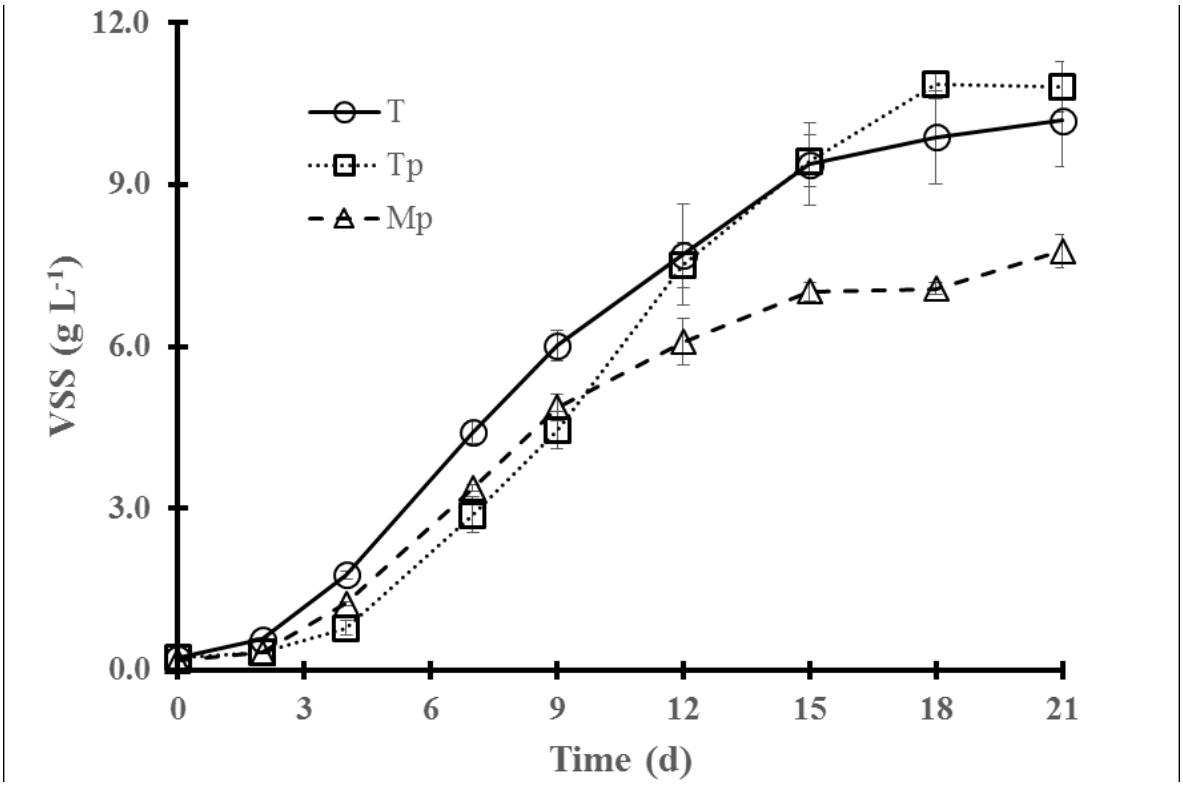



**Figure 4.1** Biomass concentration as volatile suspended solids (VSS) during the cultivation of *Scenedesmus acuminatus* in the liquid digestates from the pulp and paper wastewater treatment plant biosludge, anaerobically treated under thermophilic conditions (55 °C) without pretreatment (T), with pretreatment (121 °C) for 10 min (Tp), and under mesophilic conditions (35 °C) with pretreatment (121 °C) for 10 min (Mp).

**Table 4.2** Integrated processes of anaerobic digestion of pulp and paper industry biosludge and *Scenedesmus acuminatus* cultivation in the undiluted liquid digestates from the anaerobic digestion of the biosludge

| | Pretreatment | AD temperature (°C) | Cultivation duration (d) | Methane yield (L $CH_4$ $kg^{-1}$ VS) | Highest obtained biomass concentration (g-VSS $L^{-1}$) | Highest specific growth rate ($d^{-1}$) |
|---|---|---|---|---|---|---|
| M | No | 35 | 14 | 18[a)] | 8.8 ± 0.8[b)] | 0.99[b)] (day 4–7) |
| Mp | Yes | 35 | 21 | 101[a)] | 7.8 ± 0.3 | 0.75 (day 7–9) |
| T | No | 55 | 21 | 63[a)] | 10.2 ± 2.2 | 0.88 (day 4–7) |
| Tp | Yes | 55 | 21 | 126[a)] | 10.8 ± 1.2 | 1.02 (day 9–12) |

a) data originated from Asunis (2015)

b) microalgae were cultivated in 1.5-times diluted digestate (Tao et al., 2017)

The results of this study show that liquid digestates from pulp and paper wastewater treatment plant biosludge digestion can support high microalgal biomass yields and thus confirm the results of our previous study (Tao et al., 2017). In addition, in this study high microalgal biomass concentrations were obtained in the liquid digestates without dilution. To our knowledge, this has not been reported before. The light path in this study was not optimized, but it was shown that the color of the digestates was not a problem in the simple cultivation systems used. Thus, the microalgae should also grow well in more optimized short-path photobioreactors without dilution of the digestate. Bacteria were observed in the cultures, which was expected since the digestates were not sterilized in this study. Thus, the measured VSS values did include some bacteria associated with the microalgae. However, majority of the biomass was likely microalgae. For example, Hulatt and Thomas (2010) found an increased number of bacteria during 30-day microalgal cultivation but reported that less than 1% of carbon of the total biomass comprised of bacteria.

The influence from pulp and paper mill digestates on microalgal growth is also species-specific. For example, Kinnunen and Rintala (2016) previously reported that the highest bi-

omass concentration (less than 0.2 g-VSS $L^{-1}$) was obtained with *Scenedesmus* sp. originating from Lake Pyhäjärvi (Tampere, Finland) in 4-times diluted liquid digestate from pulp and paper industry biosludge AD after optimizing the dilution. Although the biosludge used in Kinnunen and Rintala (2016) and in this study were from the same pulp and paper mill, the different characteristics of the digestates (likely due to changes in, e.g., wood source, pulp mill operation parameters, and seasons) and microalgal strains clearly affected the obtainable biomass quantity.

#### 4.3.2.2 Nutrient removal from liquid digestates

*S. acuminatus* removed nutrients efficiently from the digestates (Figure 4.2). The ammonium concentration decreased from an initial 380–480 mg $L^{-1}$ to less than 0.2–10 mg $L^{-1}$. The ammonium removal efficiency in the thermophilic digestates was over 99.9%, which was slightly higher than that obtained in the mesophilic digestate (97.4%). The pH fluctuated between 7.8 and 8.4 (Figure S4.2 in Supplementary Material) and showed a decreasing trend likely due to ammonium uptake, which is known to reduce pH (Goldman and Brewer, 1980). The overall ammonium removal rates during the 21-day cultivation period were similar in all cultures (T: 18.3 mg $L^{-1}$ $day^{-1}$; Tp: 23.3 mg $L^{-1}$ $day^{-1}$; and Mp: 17.8 mg $L^{-1}$ $day^{-1}$). However, a clear change in the ammonium removal rate was seen in all digestates after day 7, likely due to exhaustion of phosphate and sulfate (Figure 4.2). Ammonium removal rates before and after day 7 were 43.1 and 5.9 mg $L^{-1}$ $day^{-1}$, 34.5 and 17.7 mg $L^{-1}$ $day^{-1}$, and 26.0 and 13.8 mg $L^{-1}$ $day^{-1}$ for digestate T, Tp, and Mp, respectively. This finding indicates that the exhaustion of phosphate and sulfate from the cultures could slow ammonium uptake as previously shown also by Xin et al. (2010). Several ammonium transformations (e.g., algal uptake, ammonia evaporation, bacterial growth, and nitrification) can occur in algae–bacteria consortium systems (González-Fernández et al., 2011). According to the average temperature (22 °C) and observed pH range (7.8–8.4), the theoretical fraction of unionized ammonia in all cultivations was 2.8%–10.3% (the equation used for calculation shown in Tao et al., 2017). In addition, only low levels of nitrate and nitrite (< 3 mg $L^{-1}$) were found in all cultivations. These data suggest that ammonium stripping and nitrification may have occurred, but that the main portion of the removed ammonium from the digestates was used for microbial growth.

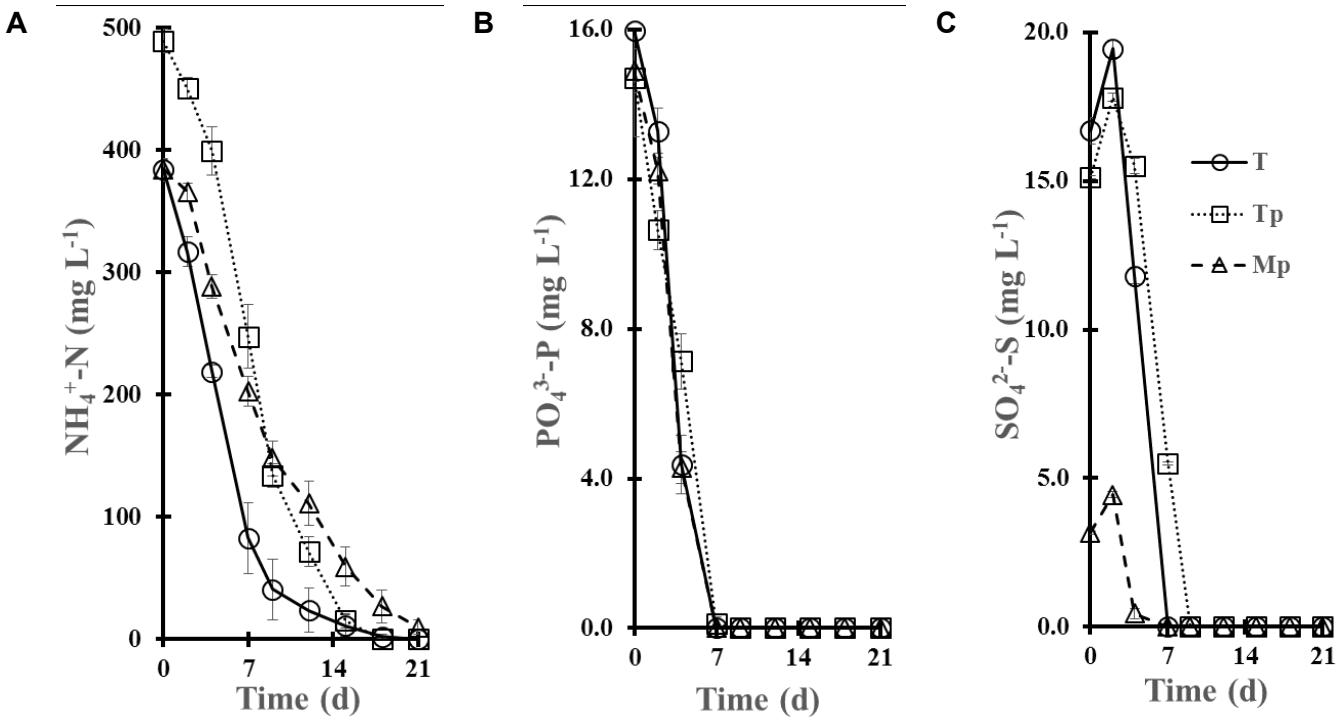



**Figure 4.2** The soluble ammonium-N (A), phosphate-P (B), and sulfate-S concentrations (C) during the cultivation of *Scenedesmus acuminatus* in the digestates from the pulp and paper wastewater treatment plant biosludge, anaerobically treated under thermophilic conditions (55 °C) without pretreatment (T), with pretreatment (121 °C) for 10 min (Tp), and under mesophilic conditions (35 °C) with pretreatment (121 °C) for 10 min (Mp). The nitrate and nitrite concentrations are not shown since they remained below 3 mg $L^{-1}$ in all cultures.

Sulfate concentration increased in all cultures from day 0 to day 2 (Figure 4.2c). The resulting sulfate likely originated from other sulfur compounds present in the digestates. During anaerobic digestion, sulfate can be converted to sulfide by sulfate-reducing bacteria, and result in the presence of $H_2S$ and $HS^-$ in the liquid phase (Cirne et al., 2008). $H_2S$ and $HS^-$ could be converted into sulfate during cultivation via chemical and biological reactions in the cultures supplied with air (Chen and Morris, 1972). Additionally, microalgae are capable of releasing enzymes that can split inorganic sulfur from organic compounds and make the sulfur available for algal growth (Giordano and Raven, 2014; Kertesz, 2000). After the initial increase, however, sulfate was completely removed by day 7-9. Phosphate removal, on the other hand, started immediately and phosphate was completely removed by day 7 in all

cultures. The overall phosphate and sulfate removal rates were 2.28 and 2.39 mg $L^{-1}$ $day^{-1}$, 1.63 and 1.68 mg $L^{-1}$ $day^{-1}$, and 2.13 and 0.45 mg $L^{-1}$ $day^{-1}$ for digestates T, Tp, and Mp, respectively. The removal rates of both phosphate and sulfate in digestate T were the highest among all digestates. Phosphorus was likely removed from the digestates through adsorption on the microalgal surface, intracellular uptake, and precipitation (Cai et al., 2013). In the present study, VSS continued to increase even though phosphate was no longer detected from the liquid digestates after day 7, which indicates that initial phosphorus level in the digestates was high enough to support microalgal growth.

Based on the results of this study, Initial sulfate concentrations in liquid digestates could affect ammonium removal efficiency and microalgal biomass production. This hypothesis is supported by the fact that the cultivations in digestates T and Tp had similar initial sulfate concentrations (15–17 mg $L^{-1}$) that enabled over 99.9% ammonium removal and similar microalgal biomass production, while the different initial sulfate concentrations in digestates T and Mp (17 vs. 3 mg $L^{-1}$), which had similar initial ammonium concentrations, resulted in different ammonium removal efficiencies and algal biomass yield. Biological nitrogen (N) uptake is catalyzed during photosynthesis by nitrogenase, which contains iron–sulfur clusters (Zheng and Dean, 1994). A shortage of either sulfur or iron can, thus, decrease the microalgal growth rate (Kumaresan et al., 2017; Liu et al., 2008). Sulfate is a primary sulfur source for microalgae in aquatic environments, but the effect of sulfate concentration on microalgal growth has not been widely studied. Mera et al. (2016) reported that the growth of microalga *Chlamydomonas moewusii* was quite similar at sodium sulfate concentrations of 0.1–3 mM ($SO_4^{2-}$-S: 3.2–96 mg $L^{-1}$), but microalgal biomass yields were lower at higher and lower sodium sulfate concentrations. In a study by Lv et al. (2017), similar *Chlorococcum* sp. growth at $SO_4^{2-}$-S levels from 6–90.3 mg $L^{-1}$ was obtained but was much lower at 0 mg $L^{-1}$ sulfate. Due to the small number of related studies, the effect of sulfate and combined effect of iron and sulfate on microalgal growth should be further studied in the future. However, it should be also noted that other micronutrients and trace elements that were not measured in this study could have caused some differences in microalgal growth.

#### 4.3.2.3 Soluble COD, DOC, DIC, and color changes

In this study, microalgal cultivation removed soluble COD and DIC to a certain extent; 29–39% removal and 47–57% removal, respectively (Figure 4.3A, D). DOC acted somewhat contradictory to soluble COD, as the DOC level increased in the mesophilic digestate (Figure 4.3C). Soluble COD removal efficiency from the thermophilic digestates (38% and 39%) was

higher than that from the mesophilic digestate (29%). The total removed dissolved carbon (<1 g $L^{-1}$) from the digestates was lower than the total carbon present in the biomass (3.9–5.4 g $L^{-1}$), when assuming that approximately 50% of the total produced biomass is carbon (Chisti, 2008). Hence, the cultivation was mixotrophic as both organic and inorganic carbon was utilized, but mainly photoautotrophic as $CO_2$ was the main carbon source used for microalgal growth.

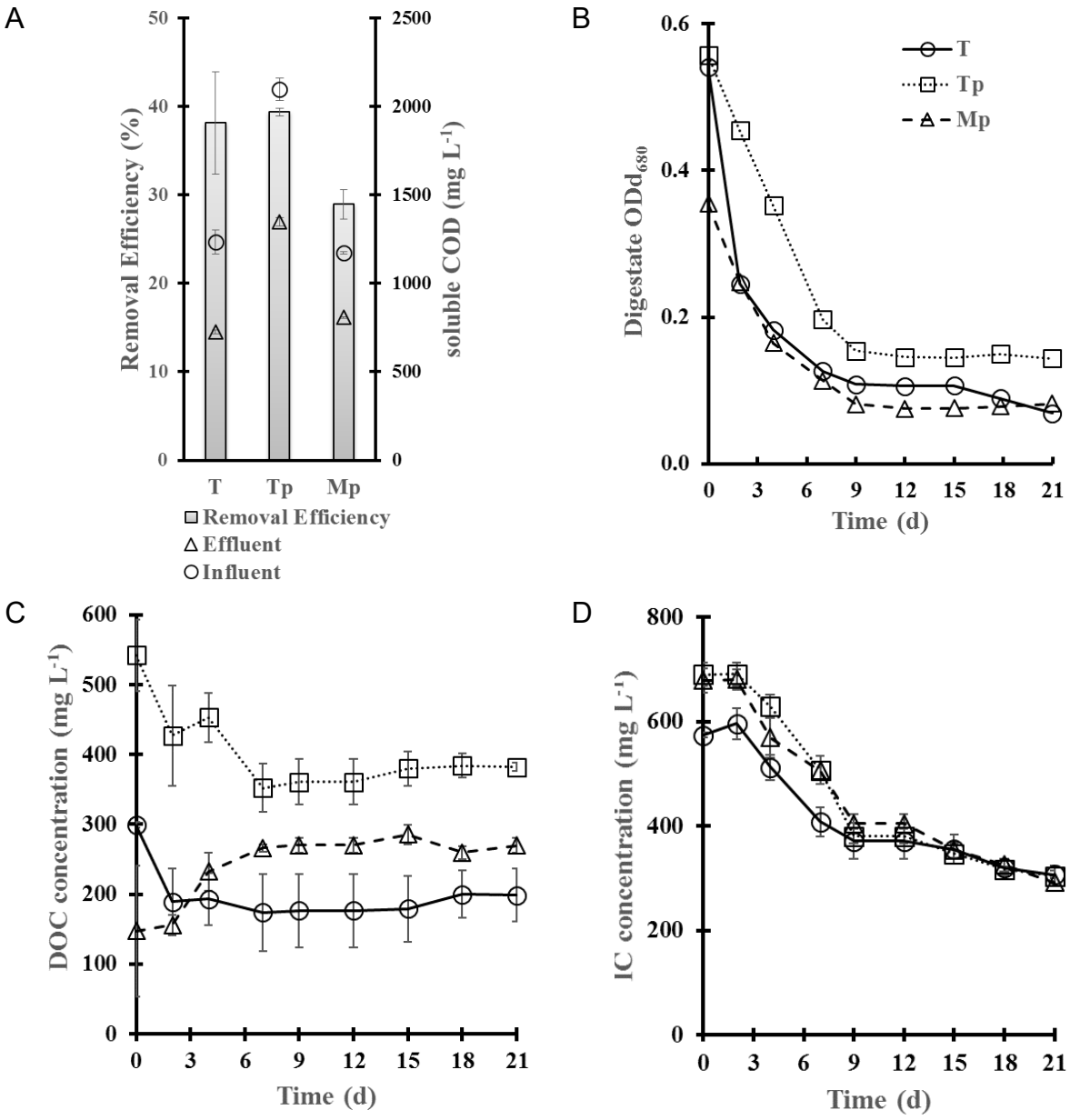



**Figure 4.3** Soluble COD concentration and removal efficiency (A), $ODd_{680}$ of the cultivation medium (B), DOC concentration (C), and DIC concentration (D) during the cultivation of *Scenedesmus acuminatus* in the digestates from the pulp and paper wastewater treatment plant biosludge, anaerobically treated under thermophilic conditions (55 °C) without pretreatment (T), with pretreatment (121 °C) for 10 min (Tp), and under mesophilic conditions (35 °C) with pretreatment (121 °C) for 10 min (Mp).

COD represents the concentration of chemical oxidizer needed to oxidize all the oxidizable organic or inorganic materials in wastewater, and DOC is used to reflect the dissolved organic carbon content of a sample. In most microalgal studies, either DOC or COD has been measured during microalgal cultivation (Eloka-Eboka et al., 2017; Guldhe et al., 2017; Wang et al., 2010), yet the correlation between COD and DOC in microalgal cultures remains unclear. For example, Marjakangas et al. (2015) reported an increase in both soluble COD and DOC concentrations, likely due to a stress caused by an initial pH decrease after *C. vulgaris* CY5 was mixotrophically cultivated in anaerobically treated piggery wastewater. Thus, it seems that changes in COD and DOC depend on growth conditions. In our study, organic carbon release from photosynthetic microalgal cells might explain the observed increase in DOC during the cultivations in mesophilic digestate. The decrease in soluble COD suggests that organic materials from the digestates were consumed during cultivation and that the amount of consumed materials was higher than the organic carbon released by the microalgae during normal photosynthetic growth. Some studies have reported relatively high COD removal efficiencies (75–80%) from liquid digestates integrated with microalgal cultivation (Yan and Zheng, 2014; Yang et al., 2015). Soluble COD in this study was not easily biodegradable and was not therefore fully removed. Further removal of soluble COD would be possible with non-biological treatments, e.g., chemical oxidation, if deemed necessary. However, further soluble COD removal would probably not be needed as the COD load (both low flow and COD concentration) from algae treatment reject waters would be minimal compared to the effluent COD load from the activated sludge plant the sludges originates, which may be up to tens of tons COD per day (e.g., Regional State Administrative Agency of Eastern Finland, 2016). Furthermore, in practice the effluent from algae treatment could be circulated to the beginning of the activated sludge process, as is typically done with dewatering reject waters after AD in municipal wastewater treatment plants.

The $ODd_{680}$ of the digestates were measured after removing the microalgae to demonstrate their color change during cultivation (Figure 4.3B). The $ODd_{680}$ values in all digestates decreased until day 9 but remained stable afterward. At the end of the batch cultivations, the color removal efficiencies in T, Tp, and Mp were 80%, 74%, and 79%, respectively. The mechanism of color removal is not clear based on the results of this study. However, Graham and Wilcox (2000) suggested that lignin (one cause of color) could be converted into other non-colored materials by microalgal metabolism. Tarlan et al. (2002) also reported that the main mechanism of color removal from pulping effluents with a mixed culture of microalgae

was metabolic conversion of colored molecules to non-colored molecules rather than adsorption. Thus, the possible reason for the lower removal efficiency of COD (29–39%) than color (74–80%) in this study was that the colored organic molecules were converted into non-colored organic molecules.

#### 4.3.2.4 Integration of methane production and microalgal cultivation in the digestate

To evaluate the different integrated AD&MC systems, the performance of each processing step is shown in an overview (treatment methods of biosludge, microalgal cultivation conditions, and bioenergy production) (Table 4.2). During the 21-day cultivation, approximately 35% more microalgal biomass (as VSS) was obtained in the thermophilic digestates than in the mesophilic digestate. This is a promising discovery, as methane production in thermophilic digestion with pretreatment was higher than that obtained in the corresponding mesophilic process; likewise, methane production in thermophilic digestion without pretreatment was also higher than that obtained in mesophilic digestion without pretreatment (Table 4.2). This finding indicates that the highest methane production and microalgal biomass yields can be obtained in the same integrated AD&MC system.

The effect of sludge pretreatment before digestion on microalgal cultivation is not, however, fully clear based on the results of this study. Asunis (2015) reported that thermal pretreatment increased the methane yield by 100% in thermophilic AD, while the increase was 460% in mesophilic AD. The difference caused by pretreatment prior to thermophilic digestion on microalgal biomass production in the digestate was not significant. Although maximum methane and microalgal biomass production were obtained with the same process (thermophilic AD with pretreatment), other factors should be considered, including the cost and energy burden of thermal pretreatment.

## 4.4 Conclusions

The cultivation of *Scenedesmus acuminatus* was successful in different undiluted digestates from pulp and paper industry biosludge treated at different AD conditions (mesophilic vs. thermophilic, with and without thermal pretreatment). *S. acuminatus* grew well (7.8–10.8 g $L^{-1}$) and removed nutrients efficiently (over 97%) from all the digestates. Color (74–80%) and soluble COD (29–39%) were partially removed. The digestates from the thermophilic

process with pretreatment generated the highest microalgal biomass concentrations, which is a promising discovery for pulp and paper industry algae-based biorefinery applications as maximum methane production was also obtained at the same conditions.

## References


1. Abeliovich, A.H.A.R.O.N., Azov, Y., 1976. Toxicity of ammonia to algae in sewage oxidation ponds. Appl. Environ. Microbiol. 31, 801-806.

2. Adamsson, M., 2000. Potential use of human urine by greenhouse culturing of microalgae (*Scenedesmus acuminatus*), zooplankton (*Daphnia magna*) and tomatoes (*Lycopersicon*). Ecol. Eng. 16, 243-254.

3. Akhiar, A., Battimelli, A., Torrijos, M., Carrere, H., 2017. Comprehensive characterization of the liquid fraction of digestates from full-scale anaerobic co-digestion. Waste Manage. 59, 118-128.

4. Arenas, E.G., Palacio, R., Juantorena, A.U., Fernando, S.E.L., Sebastian, P.J., 2017. Microalgae as a potential source for biodiesel production: techniques, methods, and other challenges. Int. J. Energy Res. 41, 761-789.

5. Asunis, F., 2015. Thermal pretreatment to enhance anaerobic digestion of pulp and paper mill biosludge (Unpublished master's thesis). Universita degli Studi di Cagliari Facolta di Ingegneria e Architettura, Italy and Tampere University of Technology, Finland. Retrieved from http://people.unica.it/giorgiadegioannis/files/2017/08/Master-Thesis-AF_17-11_v07_FINAL_FULL.pdf

6. Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: status and prospects. Renew. Sustainable Energy Rev. 19, 360-369.

7. Chen, K.Y., Morris, J.C., 1972. Kinetics of oxidation of aqueous sulfide by oxygen. Environ. Sci. Technol. 6, 529-537.

8. Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26, 126-131.

9. Cirne, D.G., Van Der Zee, F.P., Fernandez-Polanco, M., Fernandez-Polanco, F., 2008. Control of sulphide during anaerobic treatment of S-containing wastewaters by adding limited amounts of oxygen or nitrate. Rev. Environ. Sci. Bio. 7, 93-105.

10. Diniz, G.S., Silva, A.F., Araújo, O.Q., Chaloub, R.M., 2017. The potential of microalgal biomass production for biotechnological purposes using wastewater resources. J. Appl. Phycol. 29, 821-832.

11. Eloka-Eboka, A.C., Inambao, F.L., 2017. Effects of $CO_2$ sequestration on lipid and biomass productivity in microalgal biomass production. Appl. Energy 195, 1100-1111.

12. European Council. Conclusions (23 and 24 October 2014). 2030 Climate and Energy Policy Framework. EUCO 169/14; 2014. Retrieved from http://www.consilium.europa.eu/uedocs/cms_data/docs/pressdata/en/ec/145397.pdf.

13. Giordano, M., Raven, J.A., 2014. Nitrogen and sulfur assimilation in plants and algae. Aquat. Bot. 118, 45-61.

14. Goldman, J.C., Brewer, P.G., 1980. Effect of nitrogen source and growth rate on phytoplankton-mediated changes in alkalinity. Limnol. Oceanogr. 25, 352-357.

15. González-Fernández, C., Molinuevo-Salces, B., García-González, M.C., 2011. Nitrogen transformations under different conditions in open ponds by means of microalgae–bacteria consortium treating pig slurry. Bioresour. Technol. 102, 960-966.

16. Graham, L.F., Wilcox, L.W., 2000. Algae. Prentice-Hall, Englewood Cliffs, NJ.

17. Guldhe, A., Ansari, F.A., Singh, P., Bux, F., 2017. Heterotrophic cultivation of microalgae using aquaculture wastewater: A biorefinery concept for biomass production and nutrient remediation. Ecol. Eng. 99, 47-53.

18. Hulatt, C.J., Thomas, D.N., 2010. Dissolved organic matter (DOM) in microalgal photobioreactors: a potential loss in solar energy conversion?. Bioresour. Technol. 101, 8690-8697.

19. McKendry, P., 2002. Energy production from biomass (part 1): overview of biomass. Bioresour. Technol. 83, 37-46.

20. Kamali, M., Gameiro, T., Costa, M.E.V., Capela, I., 2016. Anaerobic digestion of pulp and paper mill wastes–An overview of the developments and improvement opportunities. Chem. Eng. J. 298, 162-182.

21. Kertesz, M.A., 2000. Riding the sulfur cycle–metabolism of sulfonates and sulfate esters in Gram-negative bacteria. FEMS Microbiol. Rev. 24, 135-175.

22. Kinnunen, V., Rintala, J., 2016. The effect of low-temperature pretreatment on the solubilization and biomethane potential of microalgae biomass grown in synthetic and wastewater media. Bioresour. Technol. 221, 78-84.

23. Kinnunen, V., Ylä-Outinen, A., Rintala, J., 2015. Mesophilic anaerobic digestion of pulp and paper industry biosludge–long-term reactor performance and effects of thermal pretreatment. Water Res. 87, 105-111.

24. Kumaresan, V., Nizam, F., Ravichandran, G., Viswanathan, K., Palanisamy, R., Bhatt, P., Arasu, M.V., Al-Dhabi, N.A., Mala, K., Arockiaraj, J., 2017. Transcriptome changes of blue-green algae, *Arthrospira* sp. in response to sulfate stress. Algal Res. 23, 96-103.

25. Lee, C.T., Hashim, H., Ho, C.S., Van Fan, Y., Klemeš, J.J., 2017. Sustaining the low-carbon emission development in Asia and beyond: Sustainable energy, water, transportation and low-carbon emission technology. J. Clean. Prod. 146, 1-13.

26. Liikanen, J. (2016, December 7). Biofuel from Äänekoski. Paper and Timber Journal. Retrieved from http://www.paperijapuu.fi/biofuel-from-aanekoski/

27. Liu, Z.Y., Wang, G.C., Zhou, B.C., 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresour. Technol. 99, 4717-4722.

28. Regional State Administrative Agency of Eastern Finland (2017, March 31) Environmental permit for Savon Sellu. Retrieved from https://tietopalvelu.ahtp.fi/Lupa/Lisatiedot.aspx?Asia_ID=1297010

29. Lv, J., Guo, J., Feng, J., Liu, Q., Xie, S., 2017. Effect of sulfate ions on growth and pollutants removal of self-flocculating microalga *Chlorococcum* sp. GD in synthetic municipal wastewater. Bioresour. Technol. 234, 289-296.

30. Marjakangas, J.M., Chen, C.Y., Lakaniemi, A.M., Puhakka, J.A., Whang, L.M., Chang, J.S., 2015. Simultaneous nutrient removal and lipid production with *Chlorella vulgaris* on sterilized and non-sterilized anaerobically pretreated piggery wastewater. Biochem. Eng. J. 103, 177-184.

31. Mera, R., Torres, E., Abalde, J., 2016. Effects of sodium sulfate on the freshwater microalga *Chlamydomonas moewusii*: implications for the optimization of algal culture media. J. Phycol. 52, 75-88.

32. Polishchuk, A., Valev, D., Tarvainen, M., Mishra, S., Kinnunen, V., Antal, T., Yang, B., Rintala, J., Tyystjärvi, E., 2015. Cultivation of *Nannochloropsis* for eicosapentaenoic acid production in wastewaters of pulp and paper industry. Bioresour. Technol. 193, 469-476.

33. Praveenkumar, R., Kim, B., Choi, E., Lee, K., Cho, S., Hyun, J.S., Park, J.Y., Lee, Y.C., Lee, H.U., Lee, J.S., Oh, Y.K., 2014. Mixotrophic cultivation of oleaginous *Chlorella* sp. KR-1 mediated by actual coal-fired flue gas for biodiesel production. Bioprocess Biosyst. Eng. 37, 2083-2094.

34. Rhee, G.Y., 1978. Effects of N: P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. Limnol. Oceanogr. 23, 10-25.

35. Tan, S., Yang, J., Yan, J., Lee, C., Hashim, H., Chen, B., 2017. A holistic low carbon city indicator framework for sustainable development. Appl. Energy 185, 1919-1930.

36. Tao, R., Kinnunen, V., Praveenkumar R., Lakaniemi, A.M., Rintala, A.J., 2017. Comparison of *Scenedesmus acuminatus* and *Chlorella vulgaris* cultivation in liquid digestates from anaerobic digestion of pulp and paper industry and municipal wastewater treatment sludge. J. Appl. Phycol. doi: 10.1007/s10811-017-1175-6

37. Tarlan, E., Dilek, F.B., Yetis, U., 2002. Effectiveness of algae in the treatment of a wood-based pulp and paper industry wastewater. Bioresour. Technol. 84, 1-5.

38. Xin, L., Hong-ying, H., Ke, G., Ying-xue, S., 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresour. Technol. 101, 5494-5500.

39. Veluchamy, C., Kalamdhad, A.S., 2017. Biochemical methane potential test for pulp and paper mill sludge with different food/microorganisms ratios and its kinetics. Int. Biodeterior. Biodegradation 117, 197-204.

40. Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., Ruan, R.R., 2010. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. Bioresour. Technol. 101, 2623-2628.

41. Xia, A., Murphy, J.D., 2016. Microalgal cultivation in treating liquid digestate from biogas systems. Trends Biotechnol. 34, 264-275.

42. Xu, X., Shen, Y., Chen, J., 2015. Cultivation of *Scenedesmus dimorphus* for C/N/P removal and lipid production. Electron. J. Biotechnol. 18, 46-50.

43. Yan, C., Zheng, Z., 2014. Performance of mixed LED light wavelengths on biogas upgrade and biogas fluid removal by microalga *Chlorella* sp. Appl. Energy 113, 1008-1014.

44. Yang, L., Tan, X., Li, D., Chu, H., Zhou, X., Zhang, Y., Yu, H., 2015. Nutrients removal and lipids production by *Chlorella pyrenoidosa* cultivation using anaerobic digested starch wastewater and alcohol wastewater. Bioresour. Technol. 181, 54-61.

45. Zheng, L., Dean, D.R., 1994. Catalytic formation of a nitrogenase iron-sulfur cluster. J. Biol. Chem. 269, 18723-18726.

# 5 Use of $2^2$ factorial experimental design to study the effects of iron and sulfate on growth of *Scenedesmus acuminatus* with different nitrogen sources


## Abstract

The aim of this study was to determine the combined effects of iron and sulfate on microalgal biomass concentration and removal efficiency of nitrogenous compounds by using factorial design. *Scenedesmus acuminatus* was separately cultivated in batch photobioreactors using modified N-8 media with two nitrogen sources, nitrate and ammonium. To study the interaction effect between iron and sulfate, and to reduce the total number of experimentally studied combinations, a factorial design was used. Three iron (0.1, 1, and 1.9 mg $L^{-1}$) and sulfate-sulfur concentrations (3.71, 20, and 35.8 mg $L^{-1}$) were employed to the modified N-8 media in this study. The results show that the final microalgal biomass concentration and nitrogen removal efficiency were more sensitive to the changes in iron and sulfate concentrations in the media with nitrate than with ammonium, probably because of the different assimilation mechanisms used by microalgae for these two nitrogen sources. The created models demonstrated that iron and sulfate can affect the microalgal biomass concentration and nitrate removal efficiency. However, the contributing distribution of the two variables varied – iron was statistically significant while sulfate was not. In addition, the interaction effect between iron and sulfate was not significant on microalgal biomass concentration and nitrogen removal. In synthetic medium with nitrate as nitrogen source, the highest microalgal biomass concentration was obtained with 1.0 mg $L^{-1}$ iron and 35.8 mg $L^{-1}$ sulfate-sulfur.

## 5.1 Introduction

Microalgae have gained increasing attention in the past decades as a promising feedstock for e.g. bioenergy and biofuels (for a review, see Mata et al., 2010). Many studies aiming to optimize microalgal growth have focused on the physicochemical parameters such as pH, temperature, light and medium composition (Bartley et al., 2016; Liu et al., 2008; Schnurr et al., 2016). As primary nutrients required for microalgal growth, the effects of concentration and type of nitrogen and phosphorus source on microalgal biomass production have been widely studied (Hulatt et al., 2012; Lv et al., 2018; Rhee, 1978), while other elements such as iron, magnesium, sulfur, zinc, and copper are also typically supplied to microalgal growth media to ensure sufficient growth (Mandalam and Palsson, 1998). Some metals, such as iron and magnesium are toxic to microalgae at high concentrations, but low levels (0.1–10 mg $L^{-1}$) are necessary for microalgal growth (El Baky et al., 2012; Gorain et al., 2013). Compared to the medium without iron, iron addition ($6.7*10^{-4}$, $6.7*10^{-3}$, $6.7*10^{-2}$, and $6.7*10^{-1}$ mg $L^{-1}$) enhanced the microalgal biomass production and lipid accumulation in *Chlorella vulgaris*, and the highest lipid content of 56.6% was obtained in the medium with $6.7*10^{-1}$ mg $L^{-1}$ $FeCl_3$ (Liu et al., 2008). Singh et al. (2015) reported that iron was a significant factor on enhancing lipid productivity of microalgae using response surface methodology and high iron concentration (9 mg $L^{-1}$) enabled highest lipid content of 59.6% and highest lipid productivity of 74.07 mg $L^{-1}$ $d^{-1}$ in *Ankistrodesmus falcatus* KJ671624.

Recently, the effect of sulfur has also been studied and addition of sulfate as a source of sulfur has been shown to increase microalgal biomass production as well as nitrogen and phosphorus removal efficiencies (Lv et al., 2017; Mera et al., 2016). Highest biomass concentration of *Chlamydomonas moewusii* was obtained in the medium with ammonium as nitrogen source (sulfate-sulfur concentrations: 3.2–96 mg $L^{-1}$), while the final *Chlorococcum* sp. GD biomass concentration and specific growth rates were similar in a synthetic wastewater with nitrate as nitrogen source (sulfate-sulfur: 6, 15, 25.7, 45.3 and 90.3 mg $L^{-1}$) (Lv et al., 2017; Mera et al., 2016). The highest biomass concentration was obtained in similar sulfate concentration range in both studies, however, the use of different microalgal species and cultivation conditions e.g. nitrogen source may result in different impacts of sulfate on nutrient removal and microalgal growth. For example, the nitrogen source may

play a role, because microalgae assimilate nitrate and ammonium via different assimilation mechanisms (Cai et al., 2013).

In some cases, ammonium may not be an appropriate nitrogen source for microalgae due to limited growth caused by low pH in un-buffered solutions (Hulatt et al., 2012; Lv et al., 2018) and ammonium loss via volatilization from the medium at high pH (>8.0) and temperature (Emerson et al., 1975 and Zimmo et al., 2003). However, ammonium is considered as an energetically preferred nitrogen source for microalgae (Flynn and Hipkin, 1999), because ammonium can be directly incorporated into amino acids using enzyme glutamine synthetase, while nitrate assimilation by microalgae requires two additional reduction reactions via nitrate reductase and nitrite reductase after which it is utilized as ammonium by the cells (Hellebust and Ahmad, 1989; Rowell et al., 1977; Solomonson and Barber, 1989). In the first reaction, nitrate is reduced into nitrite using nicotinamide adenine dinucleotide phosphate (NADPH) as reducing agent and ferredoxins are used to catalyze nitrite to ammonium in the second reaction (Barsanti and Gualtieri, 2014; Hellebust and Ahmad, 1989). The iron-sulfur clusters (ferredoxins) are the catalysts used during nitrate reduction, thus the combined effects of iron and sulfur on microalgal growth and nitrogen removal efficiency might vary between nitrate and ammonium assimilations.

The aim of this study was to assess the combined effects of iron and sulfate on microalgal growth and nitrogen removal efficiency using two different nitrogen sources: ammonium and nitrate. The specific research questions addressed were: (1) What is the effect of sulfate and iron on microalgal growth and nitrogen removal efficiency? (2) Is there any combined effect of iron and sulfate on microalgal growth and nitrogen removal efficiency? (3) Do the effects of iron and sulfate vary when different nitrogen sources are used? The factorial experimental design used in this study allows for greater insight into potential relationships between the factors and reveals the optimal levels of both elements, while also limiting the number of experiments required. *Scenedesmus acuminatus* was used in this study due to its high growth rates and yields observed in previous studies (Tao et al., 2017).

## 5.2 Materials and methods

### 5.2.1 Microalgal strain and medium

*Scenedesmus acuminatus* (SAG 38.81) was obtained from the SAG Culture Collection of Algae at the University of Göttingen, Germany. The stock culture was maintained in 100 mL modified N-8 medium in 250-mL Erlenmeyer flasks on an orbital shaker (150 rpm) at room temperature and at a light intensity of 40 µmol photos $m^{-2}$ $s^{-1}$. The modified N-8 medium consisted of $KNO_3$, 0.5055 g $L^{-1}$; $KH_2PO_4$, 0.7400 g $L^{-1}$; $Na_2HPO_4$, 0.2598 g $L^{-1}$; $MgSO_4 \cdot 7H_2O$, 0.0500 g $L^{-1}$; $CaCl_2 \cdot 2H_2O$, 0.0175 g $L^{-1}$; $FeNaEDTA \cdot 3H_2O$, 0.0115 g $L^{-1}$, $ZnSO_4 \cdot 7H_2O$, 0.0032 g $L^{-1}$; $MnCl_2 \cdot 4H_2O$, 0.0130 g $L^{-1}$; $CuSO_4 \cdot 5H_2O$, 0.0183 g $L^{-1}$ and $Al_2(SO_4)_3 \cdot 18H_2O$, 0.0070 g $L^{-1}$ (Praveenkumar et al., 2014). As *S. acuminatus* did not grow in the modified N-8 medium with a natural pH of 6.5, the pH was adjusted to an optimal value of 8.0 (Xu et al., 2015) by adding 5 M NaOH.

### 5.2.2 Photobioreactors

Photobioreactors used in this study consisted of a 1-L glass bottle (Pyrex) and a plastic cap with two holes serving as the gas inlet and outlet. Air at a flow rate of 0.2 L $min^{-1}$ was sparged from the bottom of the bottle by a glass distribution tube (porosity 0, ⌀ 22 mm, Duran Group, Germany) inserted to the photobioreactor through one of the holes in the cap. White fluorescent lamps (Osram L 18W/965 De Luxe Cool Daylight, Germany) were used to continuously illuminate the photobioreactors from two sides of the glass bottles with a light intensity of 240 µmol photos $m^{-2}$ $s^{-1}$, which is the optimal light intensity for *Scenedesmus dimorphus* based on Xu et al. (2015). *S. acuminatus* was inoculated from the stock cultures to the photobioreactors to provide an initial optical density of 0.27-0.29 at a wavelength of 680 nm ($OD_{680}$). The initial total culture volume in the reactors was 700 mL. The temperature of the reactors was maintained at 22±2 °C.

### 5.2.3 Experimental design and data analysis

To reveal potential interactions between the effects of sulfate and iron, a $2^2$ factorial design of experiments was used. This method allows for the selection of a minimum number of total experiments while still enabling to differentiate between the effects of individual variables

and reveal any possible interaction between the variables. In a $2^2$ factorial design, two independent variables are evaluated at two different levels.

The study started with a set of experiments (the first experimental phase) that evaluated the response surface of microalgal growth and nitrogen removal efficiency based on low (-1) and medium (+1) levels of iron and sulfate (added as $FeNaEDTA{\cdot}3H_2O$ and $Na_2SO_4$, respectively) in the media with nitrate or ammonium as nitrogen source. $KNO_3$ and $NH_4Cl$ were used as nitrogen sources in the $NO_3$ assay and the $NH_4$ assay, respectively. $MgSO_4{\cdot}7H_2O$ in the modified N-8 medium was replaced by equimolar concentrations of $MgCl_2{\cdot}6H_2O$ to keep same amount of magnesium as in the modified N-8 medium used for stock cultures while the sulfate concentration was varied. As iron and sulfate did not show a significant effect on microalgal growth or nitrogen recovery when using ammonium as the nitrogen source, the second phase of experiments was only conducted using the medium with nitrate. During the second experimental phase, wider ranges of iron and sulfate including the medium (+1) and high (+3) levels were tested with the aim to optimize biomass growth and nutrient removal efficiency. When taken together, the experiments with nitrate as nitrogen source consisted of iron and sulfate factors at low (-1), medium (+1), and high concentrations (+3) (e.g. $Fe_LS_L$ represents: low concentration of iron and low concentration of sulfate) represented in Table 5.1.

**Table 5.1** The concentrations of iron (Fe) and sulfate-sulfur (S) including low (L), medium (M) and high (H) with coded units (-1, +1, and +3) for experimental design of microalgal growth and nitrogen removal efficiency optimization.

| Factor | Concentration (coded unit) | | |
|---|---|---|---|
| | Low (-1) | Medium (+1) | High (+3) |
| Iron concentration (Fe) | 0.1 mg $L^{-1}$ | 1 mg $L^{-1}$ | 1.9 mg $L^{-1}$ |
| Sulfate-S concentration (S) | 3.7 mg $L^{-1}$ | 20 mg $L^{-1}$ | 35.8 mg $L^{-1}$ |

The low and medium levels of iron (0.1 and 1 mg $L^{-1}$) were selected based on previous studies that the highest algal growth rates were observed at added iron concentrations between 0.67 to 1.2 mg $L^{-1}$ and followed by those with added iron concentrations from 0.067–0.12 mg $L^{-1}$ (Liu et al., 2008; Ren et al., 2014). The high level of iron was set to 1.9 mg $L^{-1}$,

because iron concentrations between 2.07 and 41.4 mg $L^{-1}$ were shown to negatively affect microalgal growth (Ren et al., 2014). To minimize chemical changes from inoculum medium to modified medium, the low level of sulfate-sulfur (3.7 mg $L^{-1}$) was chosen to be the same as the sulfur concentration used in the modified N-8 medium used for stock cultures. The medium level of sulfate-sulfur (20 mg $L^{-1}$) was close to the sulfur concentrations (15–18 mg $L^{-1}$) used in previous studies in which high microalgal concentrations have been obtained (Mera et al., 2016; Tao et al., 2017). The high level of sulfate-sulfur (35.8 mg $L^{-1}$) was determined based on low and medium levels of sulfate-sulfur (Table 5.1).

The duration of the batch cultivations was 7 days in the first experimental phase, and 14 days in the second experimental phase, as stationary phase of microalgal growth was not reached and nitrogen was not completely consumed within the 7-day experiments in the first experimental phase. In the first experimental phase, water evaporated at a rate of 15 mL per day during the cultivation. To simplify the calculation of biomass concentration and nutrients concentration, distilled water was added during the second experimental phase to compensate for the evaporated water volume (marked with lines on the photobioreactors) each time before taking samples for analyses.

The measured results of microalgal biomass concentration and nitrogen removal efficiency were evaluated using R Statistical Software 3.5.1. The effects of independent factors on the dependent factors were analyzed by linear (first order models) or quadratic equations (second order models):

$$\mathrm{Y} = a_0 + a_1X_1 + a_2X_2 + a_{12}X_1X_2 \tag{5.1}$$

$$\mathrm{Y} = a_0 + a_1X_1 + a_2X_2 + a_{11}{X_1}^2 + a_{22}{X_2}^2 + a_{12}X_1X_2 \tag{5.2}$$

where Y is the response variable (microalgal biomass concentration or nitrogen removal efficiency); $a_0$ is Y-intercept; $a_1$ and $a_2$ are linear coefficients; $a_{11}$ and $a_{22}$ are the squared term coefficients; $a_{12}$ is the interaction coefficient. $X_1$ and $X_2$ are iron and sulfate-S concentrations, respectively. In the obtained models, coefficient of determination ($R^2$) or adjusted $R^2$ were used to evaluate the model fit to the experimental data (Carley et al., 2004; González-Fernández et al., 2011). P-values less than 0.05 indicated that a model term was significant for the response variable and the overall model p-values were used to choose the best model fit (González-Fernández et al., 2011).

### 5.2.4 Analytical methods

The culture pH was measured using a WTW 330 pH meter (WTW, Germany) with a Slimtrode electrode (Hamilton, Germany). The light intensity was calculated by measuring the average value of six sites on two sides of the photobioreactors' outer surface by a MQ-200 quantum meter (Apogee, USA).

#### 5.2.4.1 Determination of microalgal growth

$OD_{680}$ was measured using a Shimadzu UV-1700 Pharmaspec spectrophotometer from non-filtrated samples to assess microalgal biomass concentration. The non-filtrated samples were diluted with distilled water to give absorbance values between 0.2–0.7. Volatile suspended solids (VSS) were measured by filtering 10–15 mL of culture solution through a glass fiber filter (Whatman GF/A). Each filter containing the suspended solids was dried at 105 ºC overnight, then weighed and combusted in a 550 ºC muffle furnace for 2 h before being weighed again. VSS was determined gravimetrically as the difference between the filters after treatment at these two temperatures.

The growth rates were calculated using the following equation:

$$\mu = \frac{\ln(X_2/X_1)}{t_2 - t_1} \tag{5.3}$$

where $X_1$ is the concentration of biomass measured as VSS (g $L^{-1}$) at time $t_1$ and $X_2$ is the concentration of biomass at a time $t_2$.

#### 5.2.4.2 Determination of carbon and nutrient removal efficiency

The supernatant after VSS filtration was used for the analysis of dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), and nutrient (N, P, S) concentrations. DOC and DIC were measured with a total organic carbon analyzer (Shimadzu Model TOC-5000) with an ASI-5000 autosampler. $NH_4^+$-N was measured with an ion-selective electrode (Thermo Scientific Orion ISE meter). $NO_3^-$, $NO_2^-$, $PO_4^{3-}$, and $SO_4^{2-}$ were measured using an ICS-1600 ion chromatograph (Dionex, USA) with an AS-DV autosampler, Ion-Pac AS4A-SC anion exchange column, and ASRS-300 suppressor (2 mm). The system was operated in isocratic

mode using an eluent containing 1.9 mM $Na_2CO_3$ and 1.7 mM $NaHCO_3$, and an eluent flow rate of 1 mL $min^{-1}$.

## 5.3 Results and Discussion

### 5.3.1 The effects of iron and sulfate at low and medium levels on the microalgal biomass concentration and nitrogen removal

During the first experimental phase, the effects of iron and sulfate at low and medium levels ($Fe_LS_L$, $Fe_LS_M$, $Fe_MS_L$, and $Fe_MS_M$) on the microalgal biomass concentration and nitrogen removal efficiency were studied separately with ammonium and nitrate as the nitrogen source. The different iron and sulfate concentrations resulted in varied final microalgal biomass concentration with both nitrate and ammonium. The final microalgal biomass concentration in the $NH_4$ assay (0.83±0.15–0.99±0.02 g $L^{-1}$) was higher than that obtained in the $NO_3$ assay (0.57±0.11–0.80±0.13 g $L^{-1}$) (Figure 5.1A and B). The microalgal growth did not reach the stationary phase except that in $Fe_MS_M$ with nitrate.

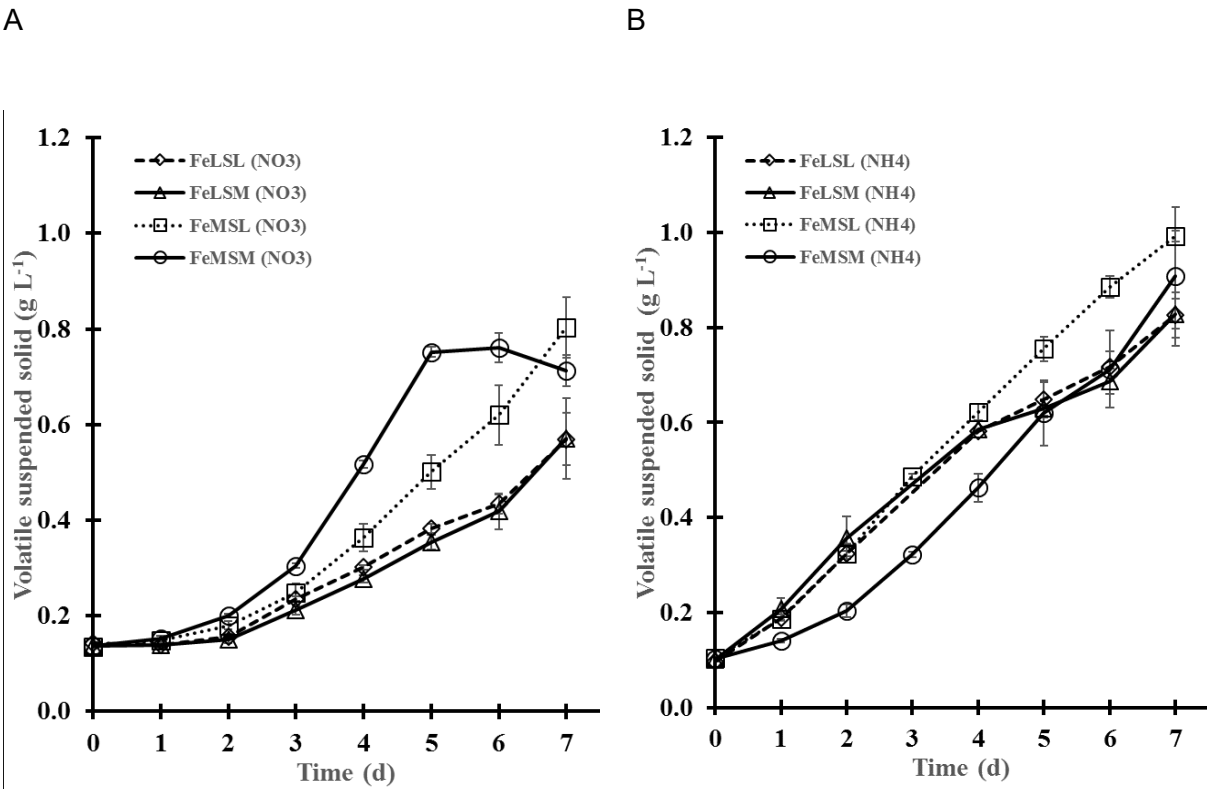



**Figure 5.1** Microalgal biomass concentration (as g-VSS $L^{-1}$) during the cultivation of *Scenedesmus acuminatus* in the modified N-8 media with different iron and sulfur concentrations (L refers to low and M refers to medium concentration of iron/sulfur) with nitrate (A) and ammonium (B) as the nitrogen source. The results of VSS are presented as the means of n = 4 (2 cultivations, 2 measurements from each); error bars represent standard deviation.

The results showed that the obtained microalgal biomass concentration was more sensitive to the changes in iron and sulfate concentration in the media with nitrate than with ammonium. Regardless of nitrogen source and sulfate concentration, the final microalgal biomass concentration increased when the iron concentration was increased. At the same iron concentration, higher microalgal biomass concentration was obtained in the medium with ammonium than with nitrate. The results of this study, thus, verify the simulations of Flynn and Hipkin (1999) who indicated via theoretical calculations that higher iron concentrations were

required in their growth simulations for nitrate assimilation to achieve the same growth rate as ammonium assimilation. However, Kim et al. (2012) obtained higher microalgal biomass concentration in a medium with nitrate than with ammonium when batch-cultivating *Chlorella sorokiniana,* while in a study by Hulatt et al. (2012), *Chlorella vulgaris* did not grow at all in a medium with ammonium due to poor buffering capacity of the medium (Hulatt et al., 2012). Thus, it seems to depend on the growth conditions and microalgal species whether the microalgae grow more efficiently with nitrate or ammonium as their nitrogen source.

In the $NO_3$ assay, the highest microalgal biomass concentration by cultivation day 5 was obtained in $Fe_MS_M$ but the biomass concentration started to decline after that. This demise was likely due to carbon limitation caused by pH increase on day 5. Culture pH in $Fe_MS_M$ of the $NO_3$ assay increased likely due to fast nitrate uptake by microalgae (Goldman and Brewer, 1980), and at high pH (e.g. >8.7) specific carbon uptake rate by microalgae decreases with increasing pH (Azov, 1982). Compared to air, mixed air with $CO_2$ could promote the microalgal growth by providing sufficient inorganic carbon source and adjusting pH in un-buffered solution.

The ammonium-N removal efficiency was higher than the nitrate-N removal efficiency after the 7-day cultivations (Figure 5.2A and B). The final nitrate-N removal efficiencies in the media with higher iron concentration ($Fe_MS_L$: 71.4%, $Fe_MS_M$: 75.1%) were higher than those in the media with lower iron concentration ($Fe_LS_L$: 43.5%, $Fe_LS_M$: 46.4%). However, the studied iron and sulfate concentrations did not significantly affect the final ammonium-N removal efficiency, which was in the similar range (82.9–93.2%) in all cultures. Also, the ammonium removal rate was very similar in all cultures with the different Fe and S concentrations (Figure 5.2B). Our results thus show that the nitrogen removal efficiency was more sensitive to the changes of iron and sulfate in the media with nitrate than with ammonium as the nitrogen source. The results are consistent to the theoretical knowledge that ferredoxins (iron-sulfur proteins) contribute more to nitrate assimilation than ammonium assimilation due to additional reduction steps of nitrate into amino acids (Hellebust and Ahmad, 1989; Rowell et al., 1977; Solomonson and Barber, 1989). In addition, Jin et al. (1998) reported that the algal nitrite reductase is a ferredoxin-dependent enzyme and the concentration of ferredoxins may be the limiting factor for nitrite reduction rate.

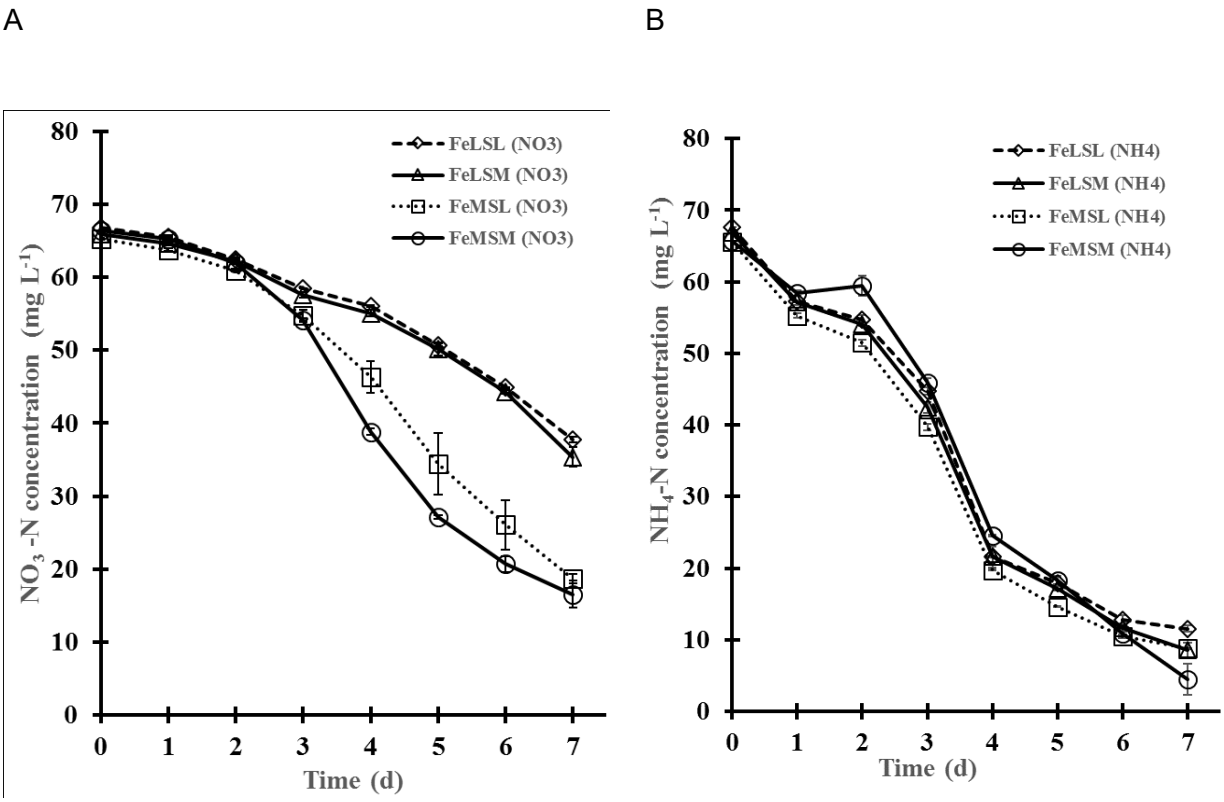



**Figure 5.2** Nitrate-N (A) and ammonium-N concentrations (B) during the cultivation of *Scenedesmus acuminatus* in the modified N-8 media ($Fe_LS_L$: low conc. of iron and low conc. of sulfur, $Fe_LS_M$: low conc. of iron and medium conc. of sulfur, $Fe_MS_L$: medium conc. of iron and low conc. of sulfur, and $Fe_MS_M$: medium conc. of iron and medium conc. of sulfur) with nitrate and ammonium as nitrogen source, respectively. The results of nitrate are presented as the means of n = 4 (2 cultivations, 2 measurements from each); error bars represent standard deviation.The results of ammonium-N are presented as the means of n = 2 (2 cultivations, 1 measurement from each); error bars represent standard error.

The measured data during the first experimental phase were used to create first order models for final microalgal biomass concentration in the $NO_3$ assay, nitrate removal efficiency, final microalgal biomass concentration in the $NH_4$ assay, and ammonium removal efficiency. However, the combined effects of iron and sulfate on ammonium removal efficiency and microalgal biomass concentration in the media with ammonium or nitrate were not clear due to overall p-values >0.05, indicating that the linear functions did not accurately describe the

relationship between iron-sulfate concentrations and ammonium removal efficiency/microalgal biomass concentration. The equation with iron and sulfate concentrations representing as united codes for the nitrate removal efficiency was:

$$NO_3\text{removal efficiency }(\%) = 58.762 + 13.838 * \text{iron} + 1.338 * \text{sulfur} \quad (5.4)$$

The p-value for the model in the $NO_3$ assay was 0.00017 (<0.001), which shows the regression analysis is statistically significant. The adjusted $R^2$=95.68% indicates that the model explaining effects of iron and sulfate on nitrate removal efficiency fits well to the experimental data. In the model, the most significant factor affecting nitrate removal efficiency was iron (p-value=0.00006) followed by sulfate-sulfur (p-value=0.282). However, the interaction effect between iron and sulfate was not a significant variable and was not therefore included in the model.

The first order models could not describe the effects of iron and sulfate on ammonium removal efficiency and microalgal biomass concentration due to the insignificant regression analyses obtained in this study likely because the data have curvature, and/or no model is suitable in the studied factors' range. However, based on the obtained first order model, sulfate concentration was not a significant factor to nitrate removal efficiency and the combined effects of iron and sulfate on the microalgal growth in the media with nitrate were not clear. Therefore, the $NO_3$ assay data in the first and second experimental phases were used to create second order models as the data set may present curvature (see details in section 5.3.2)

### 5.3.2 The effects of iron and sulfate on the microalgal biomass concentration and nitrogen removal with nitrate as nitrogen source

The second experimental phase focused on nitrate as nitrogen source with medium and high levels of iron and sulfate. The final microalgal biomass concentration on day 7 during the second phase ranged from 1.07±0.14 to 1.15±0.04 g $L^{-1}$, which were slightly higher than the results obtained in the first experimental phase (Figure S5.1B) and more than 99% nitrate was consumed by day 9 in all cultures (Figure S5.1C). Compared to all microalgal growth in the second experimental phase, a lag phase in microalgal biomass concentration of 1 day in the first experimental phase was observed (Figure S5.1B). The highest specific growth rates were observed during the second experimental phase few days earlier than

during the first experimental phase (Table 5.2). In addition, the highest specific growth rates of $Fe_MS_M$ during both phases were similar. Results of microalgal biomass concentration and nitrate removal efficiency from the first and second experimental phases were used to generate quadratic models for the $NO_3$ assays. To consider lag phase in the $NO_3$ assay and reduce the influences of nitrate deficiency on microalgal growth after day 7, the measured microalgal biomass concentrations from day 6 and day 5 were used for statistical analysis for the first and second experimental phase, respectively (Table 5.2). Meanwhile, the statistical analysis of nitrate removal efficiency was conducted using experimental results from day 5 and day 4 for the first and second experimental phases, respectively.

**Table 5.2** Comparison of experimental and predicted microalgal biomass concentration based on the model obtained using quadratic equations at different iron and sulfate concentrations in the modified N-8 media with nitrate as nitrogen source. Due to the lag phase observed during the first experimental phase, data used to create the model were taken in the $NO_3$ assay during the first and second experimental phases on day 6 and day 5, respectively.

| No. | Iron and sulfur content | Iron in coded unit | Sulfur in coded unit | Microalgal biomass concentration (g VSS $L^{-1}$) | | Maximum specific growth rate[a)] ($d^{-1}$) |
|---|---|---|---|---|---|---|
| | | | | Experimental value | Predicted value | |
| 1 | $Fe_LS_L$ | -1 | -1 | 0.434 (day 6) | 0.387 | 0.411 (day 2–3) |
| 2 | $Fe_LS_M$ | -1 | +1 | 0.418 (day 6) | 0.465 | 0.349 (day 2–3) |
| 3 | $Fe_MS_L$ | +1 | -1 | 0.620 (day 6) | 0.679 | 0.379 (day 3–4) |
| 4 | $Fe_MS_M$ | +1 | +1 | 0.761 (day 6) | 0.757 | 0.528 (day 3–4) |
| 5 | $Fe_MS_M$ | +1 | +1 | 0.815 (day 5) | 0.757 | 0.530 (day 1–2) |
| 6 | $Fe_MS_H$ | +1 | +3 | 0.828 (day 5) | 0.835 | 0.517 (day 0–1) |
| 7 | $Fe_HS_M$ | +3 | +1 | 0.740 (day 5) | 0.737 | 0.564 (day 0–1) |
| 8 | $Fe_HS_H$ | +3 | +3 | 0.805 (day 5) | 0.815 | 0.473 (day 1–2) |

a) The maximum specific growth rates were calculated according to Equation (3)

#### 5.3.2.1 Microalgal biomass concentration model with nitrate as nitrogen source

Most possible regression results of microalgal biomass concentrations are listed in Table S5.1. The model with the best fit is presented as contour plot (Figure 5.3). The quadratic polynomial equation was derived from the regression results and was the following:

$$\text{VSS}\ (\text{g}\ L^{-1}) = 0.611 + 0.146 * \text{iron} + 0.039 * \text{sulfur} - \ 0.039 * iron^2 \qquad (5.5)$$

The $p$-value for the overall model was 0.0004 (<0.05), thus the statistical relation was at 95% confidence level, which indicates that the regression analysis is statistically significant. The value of the adjusted determination coefficient $R^2$=71.1% indicates that the model can explain more than 70% of the total variation, and thus the model fits. In this study, iron ($p$=0.0006) and iron$^2$ ($p$=0.007) affected the obtainable microalgal biomass concentration more significantly than sulfur ($p$ =0.074).

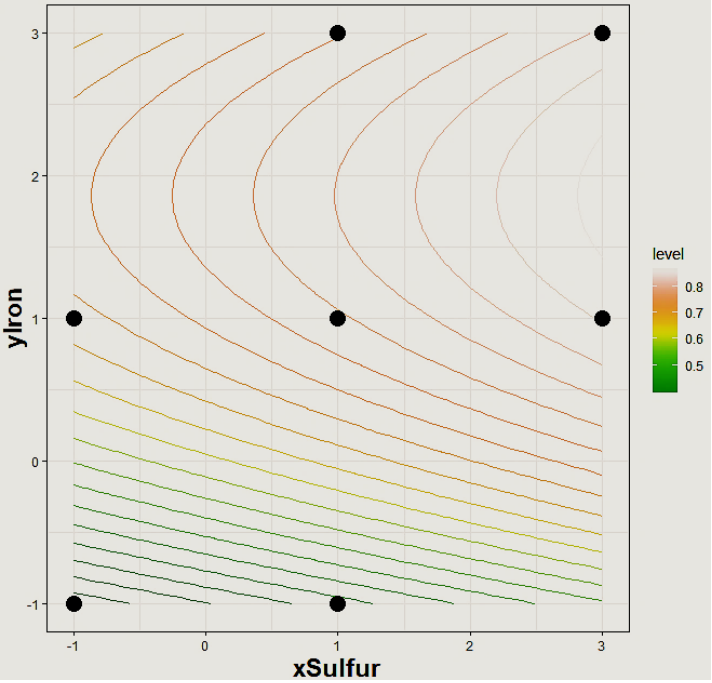



**Figure 5.3** Contour plot showing the microalgal biomass concentration as a function of iron and sulfate-sulfur. Independent variables are represented by their coded values. The different colors represent the varied levels of microalgal biomass concentration. For example, the level increases from 0.5 to 0.8 with color changing from green to orange.

#### 5.3.2.2 Nitrate removal efficiency model with nitrate as nitrogen source

Most possible regression results for the models of nitrate removal efficiency are listed in Table S4, while the model with the best fit is presented as contour plot (Fig. 4). The obtained quadratic polynomial equation for the best fit was the following:

$$NO_3\text{removal efficiency }(\%) = 43.058 + 14.446 * \text{iron} + 2.796 * \text{sulfur} - 3.469 * iron^2 - 1.169 * sulfur^2 \quad (5.6)$$

Iron ($p$ <0.0001) and iron$^2$ ($p$<0.001) significantly contributed to predicting about 84.3% of the variation in nitrate removal efficiency, while the p-value for the overall model was less than 0.0001. The order of influencing factors for nitrate removal efficiency is iron> sulfate.

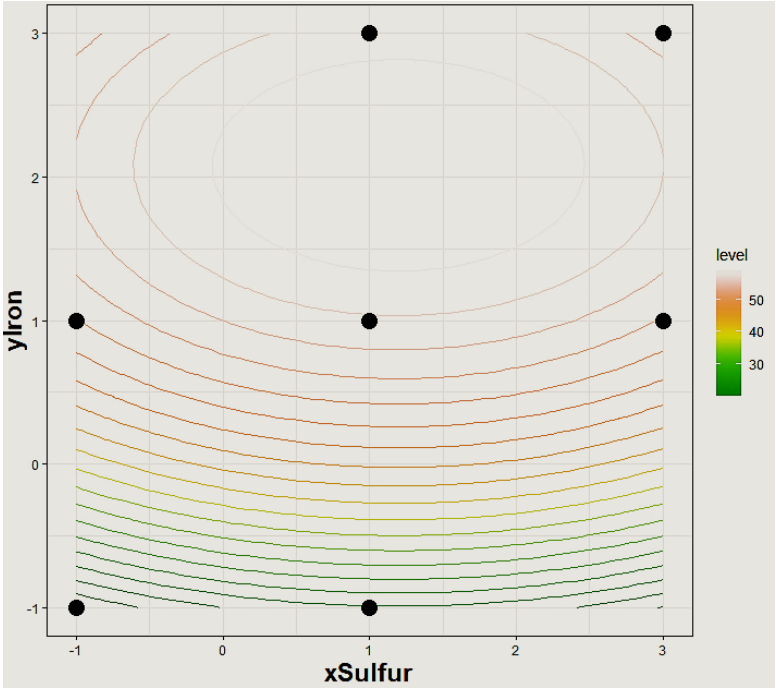



**Figure 5.4** Contour plot showing the nitrate removal efficiency as a function of iron and sulfate-sulfur. Independent variables are represented by their coded values. The different colors represent the varied levels of nitrate removal efficiency. For example, the level increases from 30 to 50 with color changing from green to orange.

#### 5.3.2.3 The effects of iron and sulfate on microalgal growth with nitrate as nitrogen source

Initial iron concentration in batch cultures was shown to have more significant effects on the microalgal biomass concentration and nitrate removal efficiency compared to sulfate-sulfur. The reason could be related to their different roles and uptake mechanisms in microalgal cells. Iron is an important trace element for microalgae because it is used in cells as a building block for many proteins (i.e. iron-sulfur proteins) required for photosynthetic electron transfer, as well as nitrogen and sulfur assimilations (Padmavathi et al., 2008; Raven, 1990). Sulfur is required for molecule production and sulfate as the major form of sulfur in nature can be taken up by microalgae (Shibagaki and Grossman, 2008).

Iron uptake by microalgae includes two pathways: a passive adsorption process on the microalgal cell surface and an active absorption process through the membrane (Cornelis and Andrews, 2010). In algal cells, sulfate is first activated with adenosine triphosphate to 5'-adenylsulfate (APS), which is then reduced to sulfite by APS reductase (Schiff and Hodson, 1970). After sulfite is reduced to sulfide by sulfite reductase (SiR), the generated sulfide is incorporated into cysteine (Schiff and Hodson, 1970). The iron-sulfur proteins consist of sulfur, which is desulfurized from cysteine, and iron (Lill and Mühlenhoff, 2008). Before the reduced sulfur is incorporated into cysteine, however, iron-sulfur proteins are also involved in sulfate assimilation to provide electrons for SiR when reducing sulfite to sulfide (Padmavathi et al., 2008). This indicates that iron as the iron-sulfur protein is involved in the sulfate assimilation, thus, the availability of iron would likely affect the sulfate assimilation in cells while iron transport in cells seemed not relate to sulfate (Giordano et al., 2008).

## 5.4 Conclusions

The varied iron and sulfate concentrations in the media with nitrate affected microalgal biomass concentration and nitrogen removal efficiency more than in media with ammonium. This was likely due to the different assimilation mechanisms used by microalgae for these two nitrogen species. Iron and sulfate concentrations affected the microalgal biomass concentration and nitrate removal efficiency, whilst iron was the only statistically significant fac-

tor. The models describing combined effects of iron and sulfate on microalgal biomass concentration and nitrate removal efficiency, which fitted best the experimental data, did not indicate any interaction effect between iron and sulfate. In the medium with nitrate as the nitrogen source, the highest final microalgal biomass concentration was obtained with initial iron and sulfate-sulfur concentrations of 1.0 mg $L^{-1}$ and 35.8 mg $L^{-1}$, respectively.

## References


Azov, Y., 1982. Effect of pH on inorganic carbon uptake in algal cultures. Applied and Environmental Microbiology, 43(6), 1300-1306.

Barsanti, L., Gualtieri, P., 2014. Algae: anatomy, biochemistry, and biotechnology. CRC Press.

Bartley, M.L., Boeing, W.J., Daniel, D., Dungan, B.N., Schaub, T., 2016. Optimization of environmental parameters for *Nannochloropsis salina* growth and lipid content using the response surface method and invading organisms. Journal of Applied Phycology, 28(1), 15-24.

Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleira, L.A., 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, 76(5), 965-977.

Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: status and prospects. Renewable and Sustainable Energy Reviews, 19, 360-369.

Cornelis, P., Andrews, S.C. eds., 2010. Iron uptake and homeostasis in microorganisms. Horizon Scientific Press.

El Baky, H.H.A., El-Baroty, G.S., Bouaid, A., Martinez, M. and Aracil, J., 2012. Enhancement of lipid accumulation in *Scenedesmus obliquus* by optimizing $CO_2$ and $Fe^{3+}$ levels for biodiesel production. Bioresource Technology, 119, 429-432.

Emerson, K., Russo, R.C., Lund, R.E. and Thurston, R.V., 1975. Aqueous ammonia equilibrium calculations: effect of pH and temperature. Journal of the Fisheries Board of Canada, 32(12), 2379-2383.

Flynn, K.J. and Hipkin, C.R., 1999. Interactions between iron, light, ammonium, and nitrate: insights from the construction of a dynamic model of algal physiology. Journal of Phycology, 35(6), 1171-1190.

Giordano, M., Norici, A., Ratti, S., Raven, J.A., 2008. Role of sulfur for algae: acquisition, metabolism, ecology and evolution. In Sulfur metabolism in phototrophic organisms (397-415). Springer, Dordrecht.

Goldman, J.C. and Brewer, P.G., 1980. Effect of nitrogen source and growth rate on phytoplankton- mediated changes in alkalinity. Limnology and Oceanography, 25(2), 352-357.

Gonçalves, A.L., Pires, J.C. and Simões, M., 2017. A review on the use of microalgal consortia for wastewater treatment. Algal Research, 24, 403-415.

Gorain, P.C., Bagchi, S.K., Mallick, N., 2013. Effects of calcium, magnesium and sodium chloride in enhancing lipid accumulation in two green microalgae. Environmental Technology, 34(13-14), 1887-1894.

Hao, T.W., Xiang, P.Y., Mackey, H.R., Chi, K., Lu, H., Chui, H.K., van Loosdrecht, M.C., Chen, G.H., 2014. A review of biological sulfate conversions in wastewater treatment. Water Research, 65, 1-21.

Hellebust, J.A., Ahmad, I., 1989. Regulation of nitrogen assimilation in green microalgae. Biological Oceanography, 6(3-4), 241-255.

Hulatt, C.J., Lakaniemi, A.M., Puhakka, J.A., Thomas, D.N., 2012. Energy demands of nitrogen supply in mass cultivation of two commercially important microalgal species, *Chlorella vulgaris* and *Dunaliella tertiolecta*. BioEnergy Research, 5(3), 669-684.

Jin, T., Huppe, H.C., Turpin, D.H., 1998. In vitro reconstitution of electron transport from glucose-6-phosphate and NADPH to nitrite. Plant Physiology, 117(1), 303-309.

Khalid, A.A.H., Yaakob, Z., Abdullah, S.R.S. and Takriff, M.S., 2019. Analysis of the elemental composition and uptake mechanism of *Chlorella sorokiniana* for nutrient removal in agricultural wastewater under optimized response surface methodology (RSM) conditions. Journal of Cleaner Production, 210, 673-686.

Kim, S., Lee, Y., Hwang, S.J., 2013. Removal of nitrogen and phosphorus by *Chlorella sorokiniana* cultured heterotrophically in ammonia and nitrate. International Biodeterioration & Biodegradation, 85, 511-516.

Lill, R., Mühlenhoff, U., 2008. Maturation of iron-sulfur proteins in eukaryotes: mechanisms, connected processes, and diseases. Annual Review of Biochemistry, 77, 669-700.

Liu, Z.Y., Wang, G.C. and Zhou, B.C., 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresource Technology, 99(11), 4717-4722.

Lv, J., Guo, J., Feng, J., Liu, Q. and Xie, S., 2017. Effect of sulfate ions on growth and pollutants removal of self-flocculating microalga *Chlorococcum* sp. GD in synthetic municipal wastewater. Bioresource Technology, 234, 289-296.

Lv, J., Wang, X., Feng, J., Liu, Q., Nan, F., Jiao, X., Xie, S., 2018. Comparison of growth characteristics and nitrogen removal capacity of five species of green algae. Journal of Applied Phycology, 1-13.

Mandalam, R.K., Palsson, B., 1998. Elemental balancing of biomass and medium composition enhances growth capacity in high-density *Chlorella vulgaris* cultures. Biotechnology and bioengineering, 59(5), 605-611.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: a review. Renewable and sustainable energy reviews, 14(1), 217-232.

Mera, R., Torres, E., Abalde, J., 2016. Effects of sodium sulfate on the freshwater microalga *Chlamydomonas moewusii*: implications for the optimization of algal culture media. Journal of Phycology, 52(1), 75-88.

Oh-Hama, T., Miyachi, S. 1988. Chlorella, pp. 3–26. In M. A. Borowitzka and L. J. Borowitzka (eds.), Microalgal biotechnology. Cambridge University Press, Cambridge.

Raven, J.A., 1990. Predictions of Mn and Fe use efficiencies of phototrophic growth as a function of light availability for growth and of C assimilation pathway. New Phytologist, 116(1), pp.1-18.

Ren, H.Y., Liu, B.F., Kong, F., Zhao, L., Xie, G.J., Ren, N.Q., 2014. Enhanced lipid accumulation of green microalga Scenedesmus sp. by metal ions and EDTA addition. Bioresource Technology, 169, 763-767.

Rhee, G.Y., 1978. Effects of N: P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake 1. Limnology and Oceanography, 23(1), 10-25.

Rowell, P., Enticott, S., Stewart, W.D.P., 1977. Glutamine synthetase and nitrogenase activity in the blue- green alga *Anabaena cylindrica*. New Phytologist, 79(1), 41-54.

Sanz-Luque, E., Chamizo-Ampudia, A., Llamas, A., Galvan, A., Fernandez, E., 2015. Understanding nitrate assimilation and its regulation in microalgae. Frontiers in Plant Science, 6, 899

Schiff, J.A., Hodson, R.C., 1970. Pathways of sulfate reduction in algae. Annals of the New York Academy of Sciences, 175(1), 555-576.

Shibagaki, N., Grossman, A., 2008. The state of sulfur metabolism in algae: from ecology to genomics. In Sulfur metabolism in phototrophic organisms (pp. 231-267). Springer, Dordrecht.

Singh, P., Guldhe, A., Kumari, S., Rawat, I., Bux, F., 2015. Investigation of combined effect of nitrogen, phosphorus and iron on lipid productivity of microalgae *Ankistrodesmus falcatus* KJ671624 using response surface methodology. Biochemical Engineering Journal, 94, 22-29.

Solomonson, L.P., Barber, M.J., 1989. Structure-function relationships of algal nitrate reductases. Molecular and genetic aspects of nitrate assimilation/edited by John L. Wray and James R. Kinghorn.

Tao, R., Lakaniemi, A.M., Rintala, J.A., 2017. Cultivation of *Scenedesmus acuminatus* in different liquid digestates from anaerobic digestion of pulp and paper industry biosludge. Bioresource Technology, 245, 706-713.

Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y., Ruan, R., 2010. Cultivation of green algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant. Applied Biochemistry and Biotechnology, 162(4), 1174-1186.

Xu, X., Shen, Y. and Chen, J., 2015. Cultivation of Scenedesmus dimorphus for C/N/P removal and lipid production. Electronic Journal of Biotechnology, 18(1), 46-50.

Zimmo, O.R., Van der Steen, N.P. and Gijzen, H.J., 2003. Comparison of ammonia volatilisation rates in algae and duckweed-based waste stabilisation ponds treating domestic wastewater. Water Research, 37(19), 4587-4594.

# 6 Low concentration of zeolite to enhance microalgal growth and ammonium removal efficiency in a membrane photobioreactor


## Abstract

The aim of this work was to study the growth and nutrient removal efficiency of a mixed microalgal culture with and without the addition of low concentrations (0.5, 1, and 5 g $L^{-1}$ of total liquid volume in the reactor) of natural zeolite. A control test in which only zeolite was added into a similar membrane photobioreactor was also conducted. The addition of 0.5 g $L^{-1}$ zeolite to a continuous-flow membrane photobioreactor increased the microalgal biomass concentration from 0.50 to 1.17 g particulate organic carbon per L while the average ammonium removal efficiency increased from 14% to 30%. Upon microscopic inspection, microalgal cells were observed growing on the surface of zeolite particles, which indicates that zeolite can support attached microalgal growth. With higher zeolite doses inside the reactor, however, the breaking apart of added zeolite particles into finer particles dramatically increased solution turbidity, which likely was not beneficial for microalgal growth due to reduced light penetration. No positive effect of increased zeolite concentration (from 0.5 to 1 and 5 g $L^{-1}$) on biomass concentration or ammonium removal was observed in this study possibly due to the increased solution turbidity. This work shows that low doses of zeolite can be used as microcarriers to enhance microalgal biomass concentration and ammonium removal efficiency, while minimizing zeolite dose would likely reduce the turbidity effects.

## 6.1 Introduction

Integration of microalgal biomass production and wastewater treatment has attracted increasing attention because the combination enables simultanous generation of treated water, nutrient recovery, and renewable feedstock for biofuels and/or value-added products [1,2,3]. Microalgal species such as *Chlorella*, *Scenedesmus* and *Chlamydomonas* have been used in different wastewater treatment studies due to their high growth rates and nutrient (N and P) removal efficiencies [1,2]. To date, reported microalgal biomass productivity in wastewaters has ranged between 0.64 and 14.8 g (dry weight) $L^{-1}$ (wastewater volume) $day^{-1}$ [1,4,5], and studies have focused on various waste streams such as source-separated human urine [6], municipal wastewater [7] and piggery wastewater [8].

The integration of microalgae and zeolite in wastewater treatment has been proposed as a way to promote microalgal biomass production and/or enhance wastewater treatment efficiency [9–11]. Zeolite can be extracted from natural deposits or chemically synthesized from high-Si and Al containing materials (e.g. coal fly ash) [12]. Natural zeolite has been used in wastewater treatment as an effective adsorbent to remove pollutants such as ammonium and heavy metals [13,14]. Zeolite, with its microporous aluminosilicate framework structure, can exchange ions with an external medium [15]. Vasconcelos et al. [10] found that zeolite significantly enhanced growth of the marine microalga *Emiliania huxleyi* in batch cultivation, and that the chemical composition of the growth medium changed because zeolite released Mn to the medium and adsorbed metals such as Cu and Zn. Kuo [16] added natural zeolite (25 g $L^{-1}$) into a microalgal sequencing batch wastewater treatment system (hydraulic retention time, HRT=2 days) and found that the addition of zeolite increased microalgal biomass production by 48.7%, and total nitrogen and ammonium removal efficiencies by 63.4% and 9.8%, respectively.

Most studies on the combined use of zeolite and microalgae for wastewater treatment have been conducted as batch cultivations [10,11,16]. However, wastewaters are typically treated in continuous-flow systems and thus tests that simulate continuous operation are needed to understand the effects of zeolite performance on microalgal growth and wastewater treatment. Membrane photobioreactors (MPBRs) are a promising technology for microalgal cultivation as they enable biomass production and harvesting in the same unit [17,18]. In practice, several parallel reactors can be operated to accommodate continuous treatment

of wastewater and regular harvesting of the produced biomass so that while harvesting could be conducted in one reactor, the other reactors could be used to actively grow the mircoalgae for wastewater treatment. In MPBRs, the produced biomass and treated wastewater are separated by membrane filtration, which provides high-quality, low-solids effluent. Additionally, if zeolite is added to the reactor, the zeolite particles can be retained in the MPBRs as the pore size of the membranes (e.g., $10^{-4}$–10 μm) is typically much smaller than the particle size of zeolite (e.g., 0.45–6 mm) [17,19].

Large dose of zeolite has been shown to have positive effects on microalgal cultivation and wastewater treatment in continuous study by Wang et al. [11]. They recently published the work of a zeolite-amended microalgal-bacterial system in a submerged MPBR using hydrothermal liquefaction wastewater. Their results showed that addition of 50 g $L^{-1}$ zeolite in the reactor increased the biomass concentration by 67.2% as well as the ammonium removal efficiency by 15.2% when the MPBR was operated with a HRT of 5 days and a solid retention time (SRT) of 15 days. However, zeolite is a material that has economic and environmental impacts. It might be possible to decrease the zeolite dose to reduce treatment costs and environmental impacts but without changing the promotion on the microalgal biomass production and nutrient removal. Therefore, this work studied the potential of adding low concentration of zeolite (0.5–5 g $L^{-1}$) to improve microalgal production and nutrient removal in a continuous-flow MPBR treating synthetic wastewater with HRT of 24 h. Specific research questions were the following: (1) Can low concentrations of zeolite stimulate the growth of microalgae in a MPBR? (2) Will microalgae with low concentrations of zeolite enable higher nutrient removal efficiency than the system with only microalgae or only zeolite? (3) Are there any potential drawbacks caused by zeolite in microalgal MPBRs?

## 6.2 Materials and Methods

### 6.2.1 Microalgae, cultivation media and zeolite

A mixed microalgal culture with *Chlorella vulgaris* as the dominant microalga was selected for this study. *Chlorella vulgaris* was selected because the species has a high growth rate and has been widely used in wastewater treatment studies, e.g. [8,20]. A mixed culture was used as they are often more robust for wastewater treatment applications than monocultures

[21,22]. The original *Chlorella vulgaris* culture was obtained from University of Texas (UTEX) Culture Collection of Algae, USA and maintained under non-sterile conditions in modified Bold 1NV medium in a 1 L Erlenmeyer flask on a shaker (150 rpm) with continuous illumination (240 µmol $m^{-2}$ $s^{-1}$, 6400K T5 Light Bulbs). The modified Bold 1NV medium consisted of $NH_4Cl$, 0.0395 g $L^{-1}$; $NaNO_3$, 0.1875 g $L^{-1}$; $CaCl_2{\cdot}2H_2O$, 0.025 g $L^{-1}$; $MgSO_4{\cdot}7H_2O$, 0.075 g $L^{-1}$; $K_2HPO_4$, 0.075 g $L^{-1}$; $KH_2PO_4$, 0.175 g $L^{-1}$; NaCl, 0.025 g $L^{-1}$; P-IV Metal solution, 6 mL $L^{-1}$; Vitamin $B_{12}$ solution, 1 mL $L^{-1}$; Biotin Vitamin solution 1 mL $L^{-1}$ and Thiamine Vitamin solution 1 mL $L^{-1}$ [23].

The synthetic wastewater used as the feed for the MPBRs contained $C_2H_3NaO_2$, 64.1 mg $L^{-1}$ (chemical oxygen demand: 50 mg $L^{-1}$); $NH_4Cl$, 445.8 mg $L^{-1}$ ($NH_4$: 150 mg $L^{-1}$); $K_2HPO_4$, 91.7 mg $L^{-1}$ ($PO_4$: 50 mg $L^{-1}$); MgSO4·$7H_2O$, 51.3 mg $L^{-1}$; $FeCl_3$, 5.8 mg $L^{-1}$ and tap water [24]. Unsterilized tap water was used in the preparation of the cultivation medium as it is a more cost-effective water source than distilled water. The use of tap water likely introduced low concentrations of trace elements as well as chlorine, nitrate and microbes present in the local tap water to the MPBRs [25]. The feed was mixed during initial preparation but was not mixed during the operation of the MBPRs. Fresh feed was prepared every 2 to 5 days depending on the number of reactors in operation and the feed tank was emptied and rinsed with tap water before adding the new feed. Chemical speciation of ions in the feed was estimated using Visual MINTEQ (Visual MINTEQ Version 3.1, https://vminteq.lwr.kth.se/) to predict possible presence of precipitates. In the theoretical speciation analysis, the average temperature (24 °C) and pH (7.8) of feed were used and the calcium concentration of tap water was assumed as 40 mg $L^{-1}$ as calcium is generally present in ground water [26].

Clinoptilolite was chosen as the zeolite type for this study because it is the most abundant natural zeolite and is used extensively all over the world [27]. The chemical composition of the zeolite (Chem-Sorb Filter Media 1/2 Cu Ft Box) as reported by the supplier is shown in Table 6.1. Zeolite with particle size of 0.5–1.0 mm has been found to result in the highest ammonium exchange capacity [14] and the particle size of 0.4–1.4 mm has been used in previous hybrid microalgal and zeolite photobioreactors [11,16]. Therefore, the clinoptilolite selected for this study was sieved to a particle size of 0.71–0.85 mm. The zeolite was rinsed several times with distilled water to remove dust and dried at 105 °C for 24 h. After cooling in the desiccator, the dried zeolite was stored at room temperature prior to the experiments.

**Table 6.1** Chemical composition of the natural zeolite (clinoptilolite) used in this study (as reported by the supplier)

| Chemical constituent | $SiO_2$ | $Al_2O_3$ | $Fe_2O_3$ | CaO | MgO | $Na_2O$ | $K_2O$ | MnO | $TiO_2$ |
|---|---|---|---|---|---|---|---|---|---|
| Mass percentage (%) | 66.7 | 11.48 | 0.9 | 1.33 | 0.27 | 3.96 | 3.42 | 0.025 | 0.13 |

### 6.2.2 Membrane photobioreactors (MPBRs) and batch tests

#### 6.2.2.1 The MPBRs setup and operation

Two MPBRs consisting of a photobioreactor and a sidestream membrane module, having a total liquid volume of 2.25 L, were used in this study (Figure 6.1). One MPBR (algal MPBR) was used to study microalgal cultivation and nutrient removal with and without zeolite addition while the other reactor (control MPBR) was used as reference with zeolite addition, but without microalgae innoculation. Two identical borosilicate glass columns were used as the photobioreactors. Each column was fitted with one external 5.2 mm diameter tubular polyvinylidene fluoride (PVDF) ultrafiltration (UF) membrane module (Pentair, X-Flow) with a nominal pore size of 0.03 µm and a membrane area of 0.017 $m^2$. The MPBRs were continuously aerated with air using cylinder aquarium air stones from the bottom at a flow rate of 0.8 L $min^{-1}$. Each membrane was backwashed with permeate for 10 s every 15 min of operation. The culture from the photobioreactor was recycled to the membrane module at a flow rate of 0.38 L $min^{-1}$. The MPBRs were operated at 24±2 °C and continuously illuminated using white fluorescent lamps (6400K T5 Light Bulbs) with a light intensity of 144 µmol photos $m^{-2}$ $s^{-1}$. Synthetic wastewater was automatically pumped into the MPBRs from the top of the photobioreactor units where a float switch sensor detected the culture level of each MPBR. The HRT of the MPBRs was 24 h and the permeate was pumped out through the membrane module (cross flow velocity: 0.3 m $s^{-1}$ and operating flux: 6.8 L $h^{-1}$ $m^{-2}$) at a rate of 2.3 mL $min^{-1}$. The mixed-phase pressure of the feed, concentrate, and permeate tubes connected to the membrane modules were monitored and recorded separately using Onset data loggers, and were read out with HOBOware software (Onset Computer Corporation,

MA, USA). These three pressure readings were then used to calculate the transmembrane pressure (TMP) of the membrane unit using the following equation:

$$P = \frac{P_f + P_c}{2} - P_p \tag{6.1}$$

where P stands for transmembrane pressure, and $P_f$, $P_c$, and $P_p$ stand for feed-side pressure, concentrate-side pressure, and permeate-side pressure, respectively.

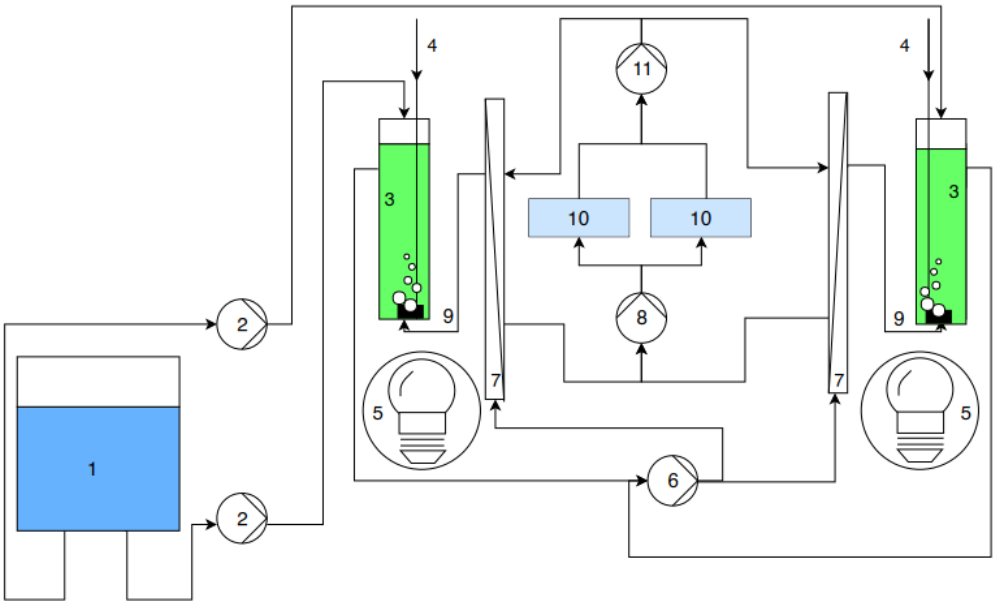



**Figure 6.1** Membrane photobioreactors used for microalgal cultivation and wastewater treatment: feed tank (1), pumps for influent feeding (2), photobioreactors (3), synthetic air inlet (4), fluorescent lamps (5), peristaltic pump with two channels to transfer cultures from photobioreactors to membranes (6), membrane modules (7), peristaltic pump with two channels to transfer permeate (8), recirculation of solution (9), permeate storage tanks (10), peristaltic pump with two channels for backwashing (11).

The algal MPBR was inoculated with the *C. vulgaris*-dominated mixed microalgal culture to an initial optical density ($OD_{680}$) of 0.2. At first, the microalgae were cultivated without zeolite (days 0-23, Phase I), subsequently 0.5 g $L^{-1}$ (total liquid volume in the reactor) zeolite was

added on day 24, (days 24-39, Phase II), zeolite concentration was increased to 1 g $L^{-1}$ on day 40 by adding 0.5 g $L^{-1}$ of zeolite (days 40-55, Phase III), and to 5 g $L^{-1}$ on day 56 by adding 4 g $L^{-1}$ of zeolite (days 56-71, Phase IV). The zeolite concentrations were chosen based on previously published batch growth studies, where higher or similar microalgal biomass production was obtained at the zeolite concentrations ranging from 0.5 to 5 g $L^{-1}$ compared to the zeolite concentrations lower than 0.5 g $L^{-1}$ or higher than 10 g $L^{-1}$ [9,28,29]. The five phases and control reactor conditions are summarized in Table 6.2.

**Table 6.2** Zeolite concentrations of the algal MPBR and the control MPBR during each experimental phase.

| Reactor | Phase | Time (days) | Zeolite (g $L^{-1}$) |
|---|---|---|---|
| algal MPBR | Phase I | 0–23[a)] | 0 |
| | Phase II | 24–39[a)] | 0.5 |
| | Phase III | 40–55[a)] | 1.0 |
| | Phase IV | 56–71[a)] | 5.0 |
| | Phase V | 72–108[a)] | 5.0 |
| control MPBR | Phase I-2 | 0-14[b)] (33–47)[a)] | 0.5 |
| | Phase II-2 | 15-32[b)] (48–65)[a)] | 1.0 |
| | Phase III-2 | 33-48[b)] (66–81)[a)] | 5.0 |

a) Time counted from the day the algal MPBR was started
b) Time counted from the day the control MPBR was started

One harvesting event of the algal MPBR occurred on day 72 in order to remove accumulated biomass from the reactor. During the harvesting event, 1.15 L of partially settled reactor content was removed from the reactor. The sampling location was located approximately 12.7 cm from the base of the reactor where circulation occurs. During normal operation, most of the zeolite settled at the bottom of the reactor, while a smaller fraction remained

suspended in the algal MPBR due to agitation caused by aeration and solution recycling. Before the harvesting event, all pumps were stopped to let the biomass settle for 5 min (to be consistent with the settling time in batch tests) to reduce zeolite loss. No zeolite was added to the algal MPBR after harvesting. In previous continuous algae-based wastewater studies, a short SRT (4–15 days) was selected to enhance the nitrogen removal rate [11,30]. However, frequent biomass harvesting would have reduced the zeolite concentration by removing the lighter, suspended fraction of zeolite. Thus, to reduce zeolite loss from the system, biomass in the MPBRs was not removed apart from sampling, and SRT can be assumed as infinity before the harvesting event and subsequently during Phase V.

The nutrient removal efficiency of zeolite was studied in the control MPBR with different zeolite concentrations for 48 days to test the performance under the same condition as the algal MPBR, but without microalgae. First, 0.5 g $L^{-1}$ zeolite was added to the control MPBR (days 0–14, Phase I-2), the zeolite concentration was increased to 1 g $L^{-1}$ on day 15 (days 15–32, Phase II-2), and to 5 g $L^{-1}$ on day 33 (days 33–48, Phase III-2). The control MPBR operation was started later than the algal MPBR operation, and the day 0 for the control MPBR corresponds to day 33 for the algal MPBR as indicated in Table 6.2.

#### 6.2.2.2 Batch tests for zeolite effects on turbidity

To determine the amount of turbidity contributed to the bulk solution via the breakdown of zeolite, batch tests were conducted in 125-mL Erlenmeyer flasks with just zeolite and water. Zeolite (0.5 g) was added to 100 mL of distilled water and the flasks were agitated on a shaker for 16 days (150 rpm, 24±2 °C). Distilled water was used in this experiment to minimize turbidity changes that might occur due to ion exchange or adsorption of ions to zeolite. For measurements, all flasks were removed from the shaker and allowed to settle for 5 min prior to any turbidity measurment. The 5 minute time interval was selected as most large zeolite particles were observed to settle within that timeframe. After settling, a 15 mL aliquot containing only the suspended zeolite particles was taken from the top of the solution and a turbidity measurement was taken of this sample. After this first measurement, the sample was poured back into the flask and the flask was stirred manually for 5 sec. A second sample of 15 mL was then taken from the middle of the solution immediately after mixing. The turbidity measurement from this second sample was then taken to determine the turbidity of the solution with all particulates. All the batch tests were conducted in triplicate.

### 6.2.3 Analytical methods

Optical density (OD) of the culture was measured at 680 nm wavelength with a Hach DR 4000 UV/VIS spectrophotometer after appropriate dilution with distilled water to give absorbance values between 0.2–0.7. It should be noted that in the algal MPBR $OD_{680}$ measurements included both biomass and zeolite, thus OD was not used as a conclusive measurement for algal growth. Feed and permeate pH were measured with a Corning pH/ion analyzer 350. Solution turbidity in the batch tests was measured with a Hach 2100P turbidimeter. The average light intensity of the MPBRs was measured from six points along the sides of the photobioreactors' outer surface. The total nitrogen (TN) (HACH method #10072), nitrate-nitrogen (HACH method #10020), ammonia-nitrogen (HACH method #10031) and total phosphates ($PO_4$) (HACH method #10127) of the synthetic wastewater and the permeate were analyzed non-filtered samples with a Hach DR 4000 UV/VIS Spectrophotometer using Hach kits according to manufacturer's instructions.

A Total Organic Carbon Analyzer (Shimadzu) equipped with a SSM-5000A solid sample module (SSM) and a non-dispersive infrared detector was used for the analysis of total organic carbon (TOC) of the feed and the permeate. The same instrument was also used to determine the particulate organic carbon (POC) of the culture in the algal MPBR. The POC concentration of the culture was used as an indicator for microalgal biomass as zeolite does not contain organic carbon. For POC analysis, 3–5 mL reactor content were filtered through 25 mm diameter Whatman GF/C glass fiber filter (1.2 µm nominal pore size). The solids retained on the filters were oven-dried for 5 min at 105 °C and cooled down in the desiccator before placing in the SSM for POC analysis.

Scanning Electron Microscopy (SEM) with Energy Dispersive X-Ray Analysis (EDX) was used to compare used zeolite from the algal MPBR with raw zeolite to determine chemical composition changes of the zeolite. SEM-EDX was performed using a JSM-6010LA InTouchScope™ (JEOL, Japan) equipped with an energy dispersive X-ray spectrometer (EDX-PGT Ge-detector) operating at 10 and 20 kV beam voltage. The analyses conducted on zeolite in this study included Secondary Electron Imaging (SEI), Backscattered Electron Composition Imaging (BEC), and Energy Dispersive x-ray Spectroscopy (EDS).

## 6.3 Results

### 6.3.1 Membrane photobioreactors

The MPBR inoculated with *Chlorella vulgaris*-dominated mixed culture (algal MPBR) was first operated without zeolite to determine the microalgal growth and nutrient removal without zeolite. Then, zeolite was added to algal MPBR at three different concentrations (0.5, 1 and 5 g $L^{-1}$, Phases II–IV) to observe the changes in microalgal growth and nutrient removal. Approximately 51.1% reactor content (the total culture volume of 2.25 L) was harvested from the algal MPBR once to reduce possible light limitation caused by increased suspended zeolite and biomass concentration (Phase V). The control MPBR was operated to study the system performance with zeolite but without microalgae at the zeolite concentrations of 0.5, 1 and 5 g $L^{-1}$ (Phases I-2, II-2 and III-2).

#### 6.3.1.1 The effects of the zeolite on pH and microalgal biomass production

The feed pH ranged from 7.4–8.1 throughout the experiment (Figure 6.2A). In the algal MPBR, the pH of the permeate was initially 8.1 (days 0–23) but decreased to approximately 6.4 after zeolite addition (days 24–71) and dropped further to approx. 5.4 after the harvesting event (days 72–108). In the control MPBR, permeate pH varied between 7.0 and 7.4 throughout the entire operation, which was higher than in the algal MPBR with similar zeolite concentrations and slightly lower than the feed pH.

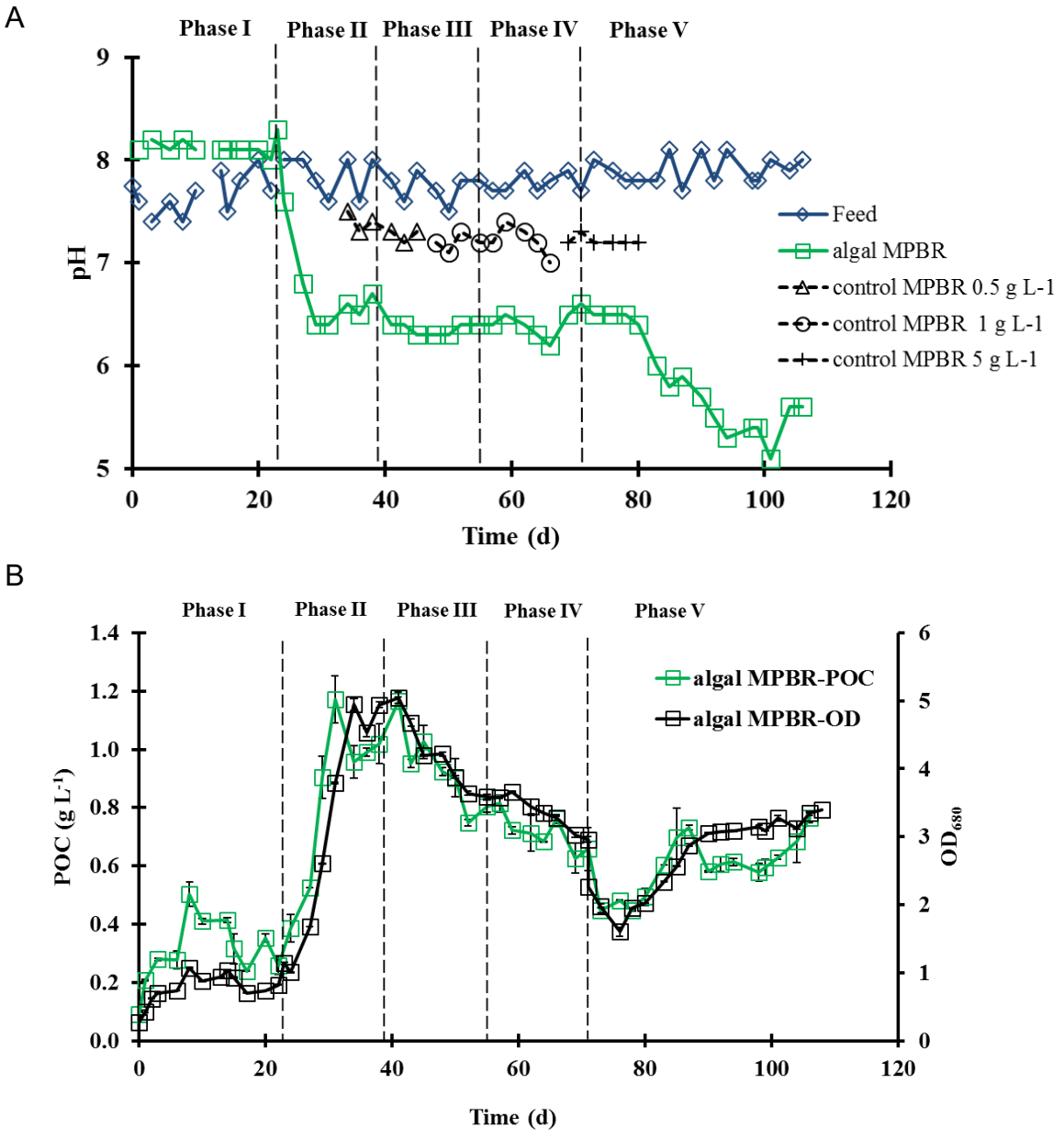



**Figure 6.2** Feed and permeate pH (A), suspended solids (optical density) and microalgal biomass concentration (particulate organic carbon) (B) during the operation of the membrane photobioreactors (algal MPBR and control MPBR). The results of $OD_{680}$ and POC are presented as the means of n = 2; error bars represent standard deviation.

The suspended solids (biomass and zeolite) in the algal MPBR was assessed with $OD_{680}$ measurements and the microalgal biomass concentration was measured as POC (Figure 6.2B). The correlations ($R^2$) of the two parameters ($OD_{680}$ and POC) were 0.83, 0.83, 0.79, 0.50, and 0.55 from Phase I to Phase V, respectively. POC increased from an initial value of 0.09 g $L^{-1}$ to 0.50 g $L^{-1}$ by day 8 (Phase I), and then decreased and stabilized at 0.3 g $L^{-1}$ for days 15–22. After zeolite was added to the algal MPBR at a concentration of 0.5 g $L^{-1}$ on day 23, an increase in the microalgal biomass concentration up to 1.17 g $L^{-1}$ was observed within 8 days (on day 31). However, subsequently POC started to decrease and levelled out to 0.6 g $L^{-1}$ even when the zeolite concentration was increased to 1 g $L^{-1}$ on day 40 and to 5 g $L^{-1}$ on day 56. On day 72, $OD_{680}$ of the algal MPBR decreased from 2.96 to 2.26 after the harvesting event and POC stabilized ranging from 0.6 to 0.7 g $L^{-1}$ after day 90 until the end of the operation of the algal MBPR.

#### 6.3.1.2 Microscopy observation of the microalgal cells and SEM-EDX analysis of microalgae and zeolite

Changes in the morphology of the microalgal cells in terms of shape during the operation of the algal MPBR were observed using an optical microscope (Figure 6.3). During Phase I, spherical-shaped *Chlorella* cells were observed, which was expected as the algal MPBR was inoculated with a *Chlorella*-dominated culture. After adding zeolite during Phases II-IV, it seems that spindle-shaped *Scenedesmus* took over the culture (Figure 6.3B, C, D). After harvesting, the share of *Chlorella* seemed to increase again and the distribution between *Chlorella* and *Scenedesmus* appeared to be more even than that in the earlier phases (Figure 6.3E).

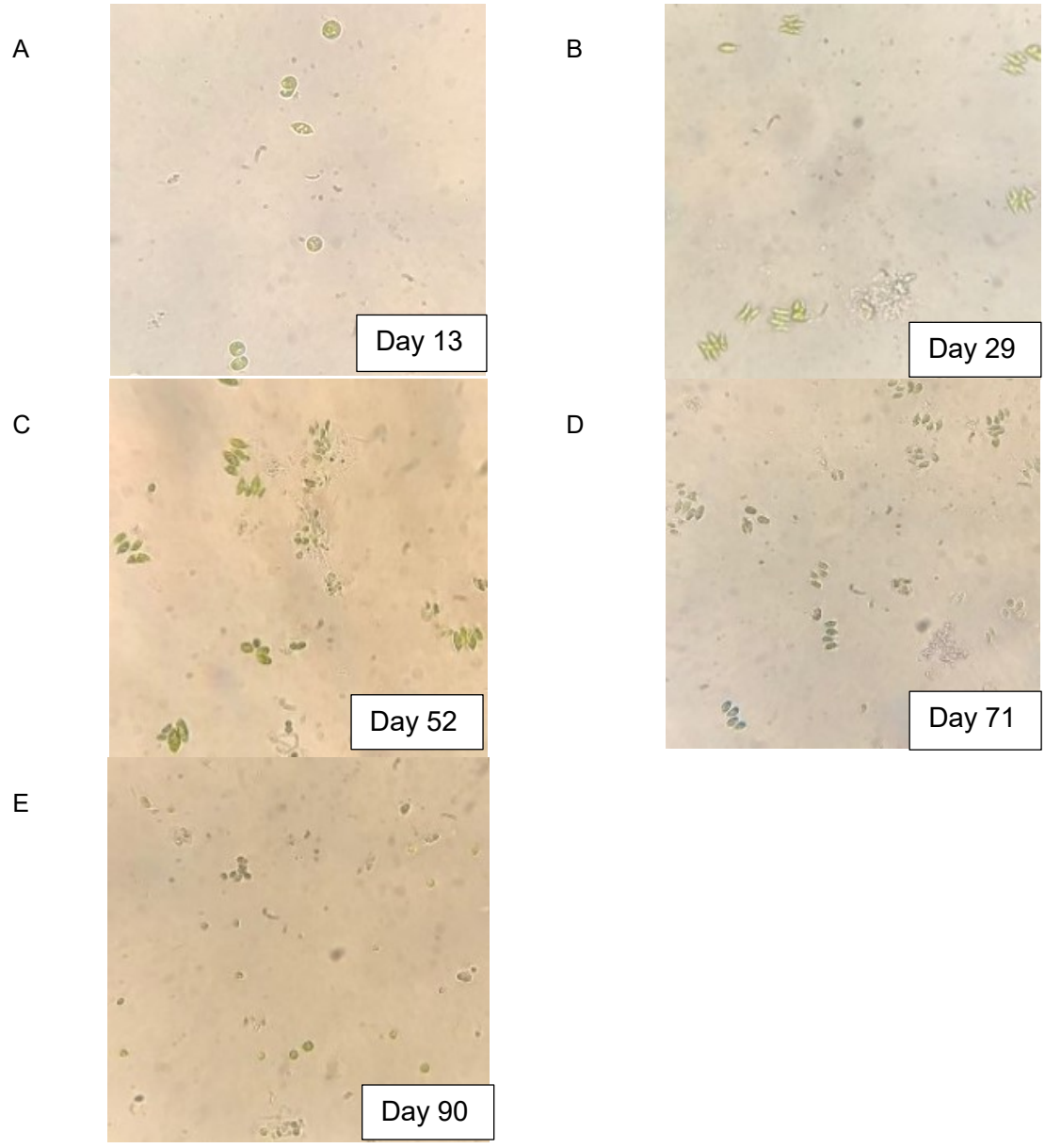



**Figure 6.3** Microscopic images demonstrating the changes in microalgal community composition in the membrane photobioreactor inoculated with *Chorella vulgaris* –dominated mixed culture (algal MPBR) on day 13 at Phase I (A), on day 29 at Phase II (B), on day 52 at Phase III (C), on day 71 at Phase IV (D), and on day 90 at Phase V (E). Magnification of 40x was used for all samples.

SEM observation and EDX analysis provided information on the structure and elemental composition of the zeolite and changes during the operation of the reactors (Figure 6.4). Used zeolite samples were collected on day 39 in the algal MPBR and day 14 in the control MPBR. The used zeolite exhibited better defined crystal structures when compared to raw zeolite (Figure 6.4A, C and D). This was likely due to shear stress encountered within the reactors, which would have helped to remove microminerals from the zeolite surface. Meanwhile in the algal MPBR, dark spindle- and spherical-shaped spots were observed on the surface of the zeolite samples. These dark spots likely originated from the microalgal suspension and were attached on the surface of the zeolite since they were not observed on the raw zeolite or zeolite samples taken from the control MPBR. EDX analysis was employed to study the elemental compositions of these spots on the zeolite (Figure 6.4H). Three points (No.1, 3 and 6 in Figure 6.4H), where the dark spots were observed, had at least two times higher carbon (35.6–47.2%) and nitrogen mass proportion (9.5–13.7%) than those (C: 11.1–22.3% and N: 0–5.4%) in other points on the zeolite (Figure S6.1 and Figure S6.2 in Supplementary Materials).

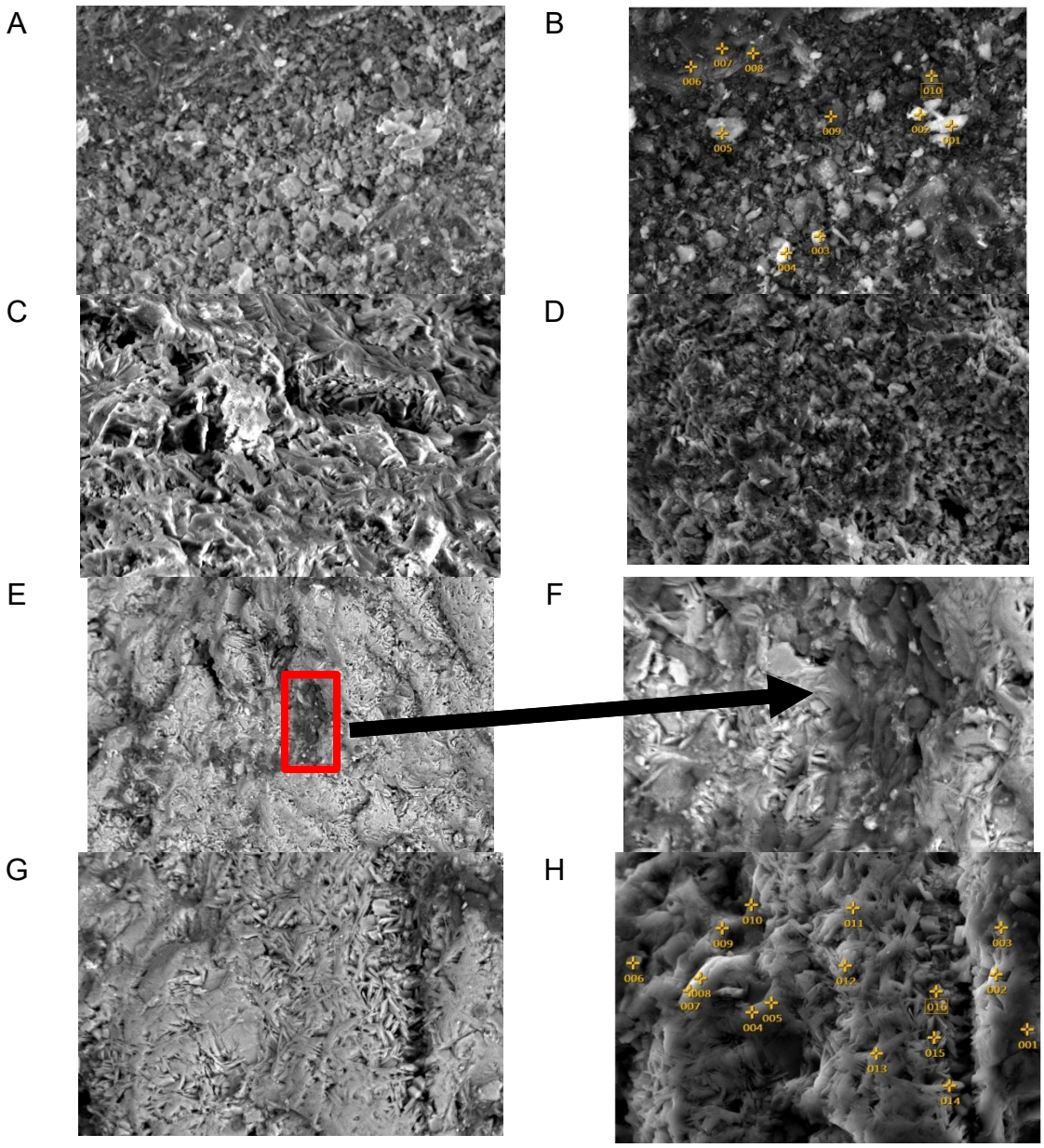



**Figure 6.4** Scanning Electron Microscopy images and Energy Dispersive X-Ray analysis from raw zeolite SEI x1500 (A), raw zeolite EDS x1500 (B), used zeolite from the algal MPBR on day 39 SEI x1500 (C), used zeolite from the control MPBR on day 14 SEI x1500 (D), used zeolite from the algal MPBR on day 108 BEC x650 (E), used zeolite from the algal MPBR on day 108 BEC x2200 (F), used zeolite from the algal MPBR on day 108 BEC x1500 (G), and used zeolite from the algal MPBR on day 108 EDS x1500 (H).

#### 6.3.1.3 The effects of the zeolite on the nutrient removal efficiency

The nutrient concentrations in the feed and permeate of the MPBRs were measured without filtering the sample before analysis (Table 6.3, Figure S6.3 in Supplementary Materials). The TN concentration of the feed was 114.9±7.0 mg $L^{-1}$ of which ammonium contributed more than 99% (114.3±5.5 mg $L^{-1}$). The TN concentration of the permeate of the algal MPBR varied between 95.8±14.5 mg $L^{-1}$ and 110.0±3.8 mg $L^{-1}$ during the whole experiment. The lowest and the highest ammonium removal efficiencies in the algal MPBR were 13.9±7.4% before zeolite addition in ~~(~~Phase I~~)~~ and 38.6±7.1% after harvesting in Phase V. The ammonium removal efficiency in the algal MPBR was similar, at approximately 30% during all three zeolite concentrations before harvesting. An average nitrate concentration of 1.0±0.5 mg $L^{-1}$ was detected in the feed, which is in accordance with the reported nitrate concentration (0.95 mg $L^{-1}$) in tap water used in the experiments [20]. The permeate nitrate concentration increased from 1.1±0.2 mg $L^{-1}$ to a maximum of 16.0 mg $L^{-1}$ after the zeolite addition and stabilized to around 5.0 mg $L^{-1}$ after day 59 until the end of the operation of the algal MBPR. In the control MPBR, total nitrogen (6.5±6.5%) and ammonium (11.5±5.2%) removal efficiencies were unstable and lower than those in the algal MPBR during the whole experiment. The average nitrate concentration in the permeate of the control MPBR was 2.5±0.9 mg $L^{-1}$ and it was lower than that in the permeate of the algal MPBR at each zeolite concentration.

**Table 6.3** The total nitrogen, ammonium-nitrogen, nitrate, and the total phosphate concentrations of the algal MPBR (Phases I–V) and the control MPBR (Phases I–III-2) permeate.

| | | algal MPBR (mg $L^{-1}$) | | | | | control MPBR (mg $L^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Phase I | Phase II | Phase III | Phase IV | Phase V | Phase I-2 | Phase II-2 | Phase III-2 |
| TN | Average | 95.8±8.5 | 96.8±4.9 | 110.0±3.8 | 102.4±8.4 | 95.8±14.5 | 109.4±8.5 | 109.3±7.2 | 107.3±12.7 |
| | Maximum | 110.8 | 103.7 | 113.3 | 114.7 | 121.5 | 119.5 | 121.0 | 122.3 |
| | Minimum | 84.5 | 91.8 | 104.5 | 91.8 | 81.0 | 98.3 | 99.3 | 85.8 |
| $NH_4^+$-N | Average | 100.8±9.0 | 80.2±4.3 | 77.0±6.3 | 80.3±3.5 | 69.3±8.0 | 100.5±2.7 | 103.5±5.0 | 97.4±8.0 |
| | Maximum | 109.6 | 86.5 | 86.4 | 84.3 | 85.5 | 104.2 | 108.2 | 105.9 |
| | Minimum | 83.1 | 75.6 | 68.0 | 73.9 | 61.2 | 96.9 | 93.4 | 84.3 |
| $NO_3^-$-N | Average | 1.1±0.2 | 5.0±2.1 | 10.9±3.7 | 4.9±0.6 | 4.5±0.5 | 3.4±1.1 | 2.2±0.5 | 2.1±0.4 |
| | Maximum | 1.5 | 7.7 | 16.0 | 6.2 | 6.0 | 4.8 | 3.2 | 2.9 |
| | Minimum | 0.7 | 1.6 | 6.6 | 4.3 | 4.0 | 2.3 | 1.7 | 1.7 |
| $PO_4^{3-}$ | Average | 5.6±5.1 | 39.0±20.0 | 59.6±7.4 | 40.3±4.9 | 27.5±5.0 | 39.6±3.9 | 30.2±3.4 | 24.0±5.7 |
| | Maximum | 14.2 | 58.3 | 70.4 | 46.8 | 41.4 | 43.2 | 34.7 | 35.1 |
| | Minimum | 0 | 3.3 | 51.8 | 34.2 | 20.4 | 34.3 | 25.9 | 17.7 |

The average phosphate concentration in the feed was 41.0±7.0 mg $L^{-1}$. Before zeolite addition in Phase I, phosphate was almost completely removed from the permeate of the algal MPBR from day 14 to day 24. Visual Minteq simulation showed that the feed (pH=7.8) likely contained chemical precipitates including $Ca_3(PO_4)_2$, $Ca_4H(PO_4)_3 \cdot 3H_2O$, $MgFe_2O_4$ (magnesioferrite), and $FePO_4 \cdot 2H_2O$ (strengite). It is likely that phophate precipitates in the feed were pumped to the MPBRs and that more precipitates were formed in the algal MPBR in Phase I, as high pH in the reactor (8.1) favored precipitation. After adding 0.5 g $L^{-1}$ of zeolite to the algal MPBR, the permeate phosphate concentration increased up to 70.4 mg $L^{-1}$, which exceeded the feed concentration indicating release of phosphate from the algal MPBR to the permeate after the pH decrease to 6.4 followed by the addition of zeolite. The permeate phosphate concentration in the algal MPBR started to decrease from Phase III to Phase V. In the control MPBR, the phosphate removal efficiencies were 4.4±7.9%, 27.5±8.3 and 42.6±16.2% from Phase I-2 to Phase III-2, respectively.

### 6.3.2 The effects of zeolite on solution turbidity in batch tests

In the algal MPBR, the correlation between $OD_{680}$ and POC being 0.83, 0.83, 0.79, 0.50, and 0.55 from Phase I to Phase V decreased with time and increased zeolite concentration. In addition, the culture color in the algal MPBR turned gray after the zeolite concentration was increased to 5 g $L^{-1}$. It was hypothesized that zeolite particles could break down into finer particles in the MPBRs, which could affect the turbidity and thus reduce light penetration within the reactor. The fine particles could also artificially contribute towards biomass content analyzed via $OD_{680}$; however, it would not affect POC analysis, which would explain the reduced correlation between $OD_{680}$ and POC. To assess the impact of zeolite on turbidity, batch tests with 5 g $L^{-1}$ zeolite a) after settling for 5 min to represent the solution with unsettled fine zeolite particles and b) after mixing to represent the solution with completely suspended zeolite particles were sampled and analyzed (Figure 6.5). The turbidities measured using both methods increased from day 1 to day 16 and were higher for the mixed solution than those taken after settling for 5 min at each sampling point.

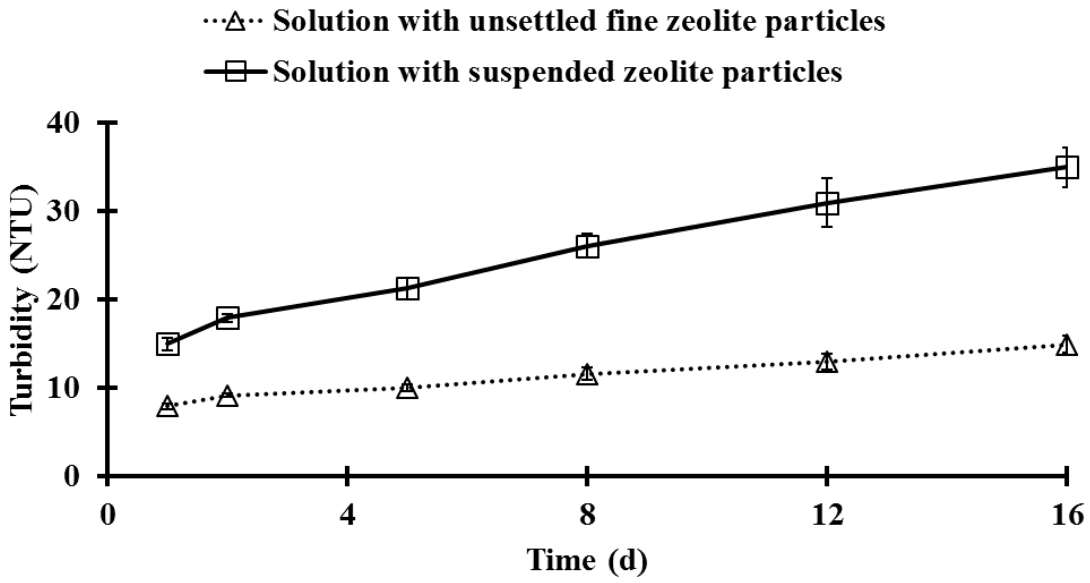



**Figure 6.5** The effects of zeolite (5 g $L^{-1}$) on solution turbidity in distilled water kept under constant shaking. The sample taken after settling for 5 min represents the solution with unsettled fine zeolite particles and the sample taken after mixing represents the solution with suspended zeolite particles of all sizes. The results of turbidity are presented as the means of n = 3; error bars represent standard deviation.

## 6.4 Discussion

This study demonstrated that higher microalgal biomass concentrations can be obtained by adding low concentrations of zeolite to a continuous-flow MPBR when compared to a similar system lacking zeolite. In the algal MPBR, the highest microalgal biomass concentration was obtained at the zeolite concentration of 0.5 g $L^{-1}$. The average microalgal biomass concentration with the presence of zeolite was 0.73 g $L^{-1}$ (Phases II-V, days 23-108), which was in fact higher than the highest microalgal biomass concentration (0.50 g $L^{-1}$) obtained on day 31 without zeolite addition. To our knowledge, this is the first study to demonstrate a microalgal biomass increase at such a low concentration of zeolite in a continuously-fed photobioreactor. In a previous study by Wang et al. [11], similar phenomena (increased microalgal biomass concentration and ammonium removal efficiency) were observed, but with significantly higher zeolite concentration of 50 g $L^{-1}$ zeolite addition has cost implications and the extraction of the raw material has energy and environmental ramifications, it is preferable to use the smallest amount of zeolte possible to achieve the desired effect.

One potential reason for the higher biomass concentration in the presence of zeolite is that zeolite provides a habitat for biofilm-based growth. Use of other carrier materials such as mohair, cotton and linen have been shown to enhance microalgal growth compared to cultivation systems relying solely on suspended growth [31]. The enhanced growth results from microalgal biofilm's increased competing ability against bacteria and protozoa [31]. Additionally, presence of the carrier materials increased retention time of $CO_2$ containing gas bubbles [31]. Wang et al. [11] suggested that microalgae could form biofilms on the zeolite surfaces when they conducted a study on zeolite-amended microalgal-bacterial culture grown in a submerged membrane photobioreactor, but they did not confirm this hypothesis by analyzing the zeolite. In this study, dark spots were observed on the surface of zeolite samples taken from the algal MPBR through SEM. The results from SEM-EDX analysis showed that the C:O mass ratios and C:N mass ratios of three measured dark spots (Fig. 4G, H) were 0.9, 1.6 and 1.1, and 3.5, 3.5 and 4.2, respectively. Based on an approximate molecular formula of microalgal biomass, $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ [32], the C:O and C:N mass ratio of microalgal biomass are 1.8 and 5.7, respectively. The C:O and C:N mass ratios of the three measured dark spots were thus in the same range as those calculated based on the theoretical biomass composition of microalgae. The slight difference of C:O and C:N ratio could be due to the variation in growth conditions. For example, the C:N ratio in microalgal cells has been shown to decrease in nitrogen limited environments where algae often begin to accumulate lipids inside their cells [33]. Thus, the shape, size and elemental composition of the spots indicate that microalgae attached to the surface of the zeolite particles [34,35].

Apart from supporting the microalgal growth as biofilm carriers, zeolite can exchange ammonium from wastewater to its surface and molecular structure [15,19], which could also contribute to the increase of microalgal biomass concentration due to higher availability of ammonium. Due to the efficient ion exchange properties of zeolite, the ammonium concentration around the zeolite particles would be higher than in the bulk solution [36]. Thus, the microalgal cells on the zeolite or around the zeolite would grow faster due to the higher availability of ammonium, as long as the ammonium concentration in the microenvironment is not high enough to inhibit the microalgal growth. The higher ammonium concentration near the zeolite might have also influenced the change in the dominant microalgal genus in the algal MPBR between Phase I and Phase II as it has been reported that *Scenedesmus* utilizes ammonium more efficiently than *Chlorella* [37,38].

Another change in the microalgal community composition was observed after harvesting 51.1% of the culture at the beginning of Phase V. After harvesting, the share of *Scenedesmus* seemed to decreased and the culture appeared to have almost even amounts of *Chlorella* and *Scenedesmus* cells based on microscopy observations. Harvesting affected the growth conditions in the reactor by

increasing light availability and decreasing the reactor pH, both of which are known to affect microalgal growth [33,39]. However, the magnitude and direction of the effects for both pH and light availability are species specific. Our previous study showed that growth of *Chlorella vulgaris* in modified N-8 medium was optimal at lower pH and light intensity (pH=6.5, 150 μmol $m^{-2}$ $s^{-1}$) compared to *Scenedesmus acuminatus* (pH=8, 240 μmol $m^{-2}$ $s^{-1}$) [38]. The increased availability of light which occurred after harvesting, likely favored the growth of *Scenedesmus* while the decreased pH favored the growth of *Chlorella* in the algal MPBR. That helps to explain why two microalgal genera appeared to be evenly matched during Phase V. In this study, the mixed culture adapted well to the changing growth conditions (e.g. pH), which ensured that despite the changing conditions the overall microalgal biomass concentrations remained comparatively high throughout the study. In practice, changing conditions are common as wastewater composition in the same system can vary widely due to operational and environmental variations [40].

This shows that the integration of microalgae and low concentrations of zeolite in a MPBR can promote higher ammonium removal efficiencies than either microalgae or zeolite alone. The increased ammonium removal rates result from a combination of ammonia adsorption to zeolite and ammonia uptake by microalgal biomass whose growth is stimulated by zeolite addition. The pH decrease observed at the beginning of Phase II in the algal MPBR occurred likely due to rapid ammonium uptake and it is known that microbial ammonium uptake can decrease pH as the reaction produces $H^+$ ions [41]. In both reactors, nitrification was observed as evidenced by higher nitrate concentrations in the permeate than in the feed. However, the concentration of nitrate produced, as mg N $L^{-1}$, was generally less than 20% of the consumed ammonium. This is in accordance with previous studies, which have reported that presence of zeolite in the membrane bioreactor enhanced the nitrification rate likely because nitrifying bacteria prefer to grow in attached form [42,43]. Compared to the control MPBR, higher nitrate concentrations were measured in the permeate of the algal MPBR because microalgae produced $O_2$ and likely excreted organic matter necessary for nitrification [44].

During Phase I in algal MPBR, the phosphate removal was primarily driven by chemical mechanisms. Visual Minteq results demonstrated the possible precipitates forming in the feed tank, which helps to explain why the measured phosphate concentration of the feed was consistently below the expected value of 50 mg $L^{-1}$ except for the days when the feed was freshly made. More precipitates formed inside the algal MPBR during Phase I since the pH in the algal MPBR (8.1) was higher than in the feed pH (7.8). The decreased pH from Phase II onwards reduced the likelihood of phosphate precipitates and resulted in solubilization of the existing precipitates. This explains the release of phosphate into the algal MPBR during Phase II. Another reason for the decrease in the formation of

precipitates was cation (e.g. $Ca^{2+}$, $Mg^{2+}$, and $Fe^{3+}$) adsorption onto the surface of zeolite, thus decreasing their availability to form precipitates. It has been reported that under neutral pH the precipitation of iron hydroxides was prevented or slowed down due to $Fe^{3+}$ adsorption by negatively charged zeolite [45]. In this study, the difference of phosphate removed by microalgal uptake before and after zeolite addition could not be quantitated due to the formation of precipitates. Theoretically, phosphate removal by microalgae uptake should have increased as the microalgal biomass increased. Additionally, luxury uptake of phosphorus, during which more phosphorus than is needed for growth is taken up by the microalgae and stored as polyphosphate, is a common occurrence in some algal communities [46]. Thus, the phosphate removal efficiency by microalgae likely increased slightly due to observed higher microalgal biomass concentration while the overall phosphate removal efficiency decreased because the conditions were less suitable for chemical removal via precipitation after zeolite addition.

SEM observations indicated that the large particles of used zeolite taken from both reactors had smoother surfaces than raw zeolite likely due to shear stress caused by aeration and solution recycling during the MPBR operation. The results of the batch test confirmed this hypothesis as zeolite was observed breaking apart in the flasks when subjected to light agitation. The increased solution turbidity resulting from the breakdown of zeolite into finer particles likely reduced light penetration within the reactor. Kasiri et al. [45] speculated a similar problem of reduced light penetration caused by increased Fe-ZSM5 zeolite dosage (0.125–1.00 g $L^{-1}$).

The results of this study indicate that the addition of low concentrations of zeolite to a MPBR can be beneficial for microalgal growth and ammonium removal. However, enhanced phosphorus removal was not observed, possibly due to the formation and subsequent dissolution of phosphorus precipitates. The results also demonstrate that separation of microalgae and zeolite can be a challenge due to their attached growth. This is problematic both for recycling the zeolite within the wastewater treatment process and for further processing of the microalgal biomass. The wastewater treatment costs would increase if zeolite becomes a consumable which needs to be replaced every time a harvesting event occurs. The presence of zeolite in the microalgal biomass can also reduce the quality of the produced biomass. For example, zeolite can adsorb heavy metals which are common in some wastewaters and which are known to be detrimental to the catalysts used in biodiesel production [47,48]. Therefore, the effects of the presence of zeolite on processing and utilization of the produced microalgal biomass and/or ways to separate microalgae from the zeolite should be studied in the future.

## 6.5 Conclusions

Microalgae and zeolite were used together to promote microalgal cultivation and synthetic wastewater treatment in a sidestream membrane photobioreactor. It was found that a low level of zeolite addition (0.5 g $L^{-1}$ of reactor) doubled the microalgal biomass concentration from 0.50 to 1.17 g POC $L^{-1}$ and increased the ammonium removal efficiency from 14% to 30%. After the zeolite addition and harvesting, microscopic images indicated changes in the microalgal community composition, which suggests that the mixed culture adapted well to the changing conditions within the reactor. The microalgae were also observed growing on the surface of the zeolite, which suggests that zeolite served as a medium for attached growth. There are a number of benefits to promoting attached algal growth, however it does make separation of the microalgae from zeolite more challenging upon harvesting. An increase in the zeolite concentration (from 0.5 to 1 and 5 g $L^{-1}$) did not result in a further increase of microalgal growth or nutrient removal efficiency, which was likely due to the increased turbidity caused by zeolite decomposition limiting light penetration within the reactor. Thus, the exact level of zeolite dosing should be optimized in algae-based wastewater treatment before practical applications. Taking cost and environmental impacts into consideration, the effects of zeolite concentrations lower than 0.5 g $L^{-1}$ should be studied in the future to ensure optimized use of the zeolite.

## References


[1] E.S. Salama, M.B. Kurade, R.A. Abou-Shanab, M.M. El-Dalatony, I.S. Yang, B. Min, B.H. Jeon, Recent progress in microalgal biomass production coupled with wastewater treatment for biofuel generation, Renew. Sust. Energ. Rev. 79 (2017) 1189-1211. https://doi.org/10.1016/j.rser.2017.05.091

[2] T. Suganya, M. Varman, H.H. Masjuki, S. Renganathan, Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: a biorefinery approach, Renew. Sust. Energ. Rev. 55 (2016) 909-941. https://doi.org/10.1016/j.rser.2015.11.026

[3] T. Cai, S.Y. Park, Y. Li, Nutrient recovery from wastewater streams by microalgae: status and prospects, Renew. Sust. Energ. Rev. 19 (2013) 360-369. https://doi.org/10.1016/j.rser.2012.11.030

[4] S. Hongyang, Z. Yalei, Z. Chunmin, Z. Xuefei, L. Jinpeng, Cultivation of *Chlorella pyrenoidosa* in soybean processing wastewater, Bioresour. Technol. 102 (2011) 9884-9890. https://doi.org/10.1016/j.biortech.2011.08.016

[5] K. Tuantet, H. Temmink, G. Zeeman, M. Janssen, R.H. Wijffels, C.J. Buisman, Nutrient removal and microalgal biomass production on urine in a short light-path photobioreactor, Water Res. 55 (2014) 162-174. https://doi.org/10.1016/j.watres.2014.02.027

[6] S. Jaatinen, A.M. Lakaniemi, J. Rintala, Use of diluted urine for cultivation of *Chlorella vulgaris*, Environ. Technol. 37 (2016) 1159-1170. https://doi.org/10.1080/09593330.2015.1105300

[7] P.J. McGinn, K.E. Dickinson, K.C. Park, C.G. Whitney, S.P. MacQuarrie, F.J. Black, J.C. Frigon, S.R. Guiot, S.J. O'Leary, Assessment of the bioenergy and bioremediation potentials of the microalga *Scenedesmus* sp. AMDD cultivated in municipal wastewater effluent in batch and continuous mode, Algal Res. 1 (2012) 155-165. https://doi.org/10.1016/j.algal.2012.05.001

[8] J.M. Marjakangas, C.Y. Chen, A.-M. Lakaniemi, J.A. Puhakka, L.M. Whang, J.S. Chang, Simultaneous nutrient removal and lipid production with *Chlorella vulgaris* on sterilized and non-sterilized anaerobically pretreated piggery wastewater, Biochem. Eng. J. 103 (2015) 177-184. https://doi.org/10.1016/j.bej.2015.07.011

[9] G. Markou, D. Vandamme, K. Muylaert, Using natural zeolite for ammonia sorption from wastewater and as nitrogen releaser for the cultivation of *Arthrospira platensis*, Bioresour. Technol. 155 (2014) 373-378. https://doi.org/10.1016/j.biortech.2013.12.122

[10] M.T.S. Vasconcelos, J.L. López-Ruiz, A. Garcia, M.F.C. Leal, A. Fachini, Effect of zeolites on cultures of the marine micro-algae *Emiliania huxleyi*, Aquacult. Eng. 31 (2004) 205-219. https://doi.org/10.1016/j.aquaeng.2004.04.001

[11] M. Wang, L. Schideman, H. Lu, Y. Zhang, B. Li, W. Cao, Zeolite-amended microalgal-bacterial system in a membrane photobioreactor for promoting system stability, biomass production, and wastewater treatment efficiency to realize Environmental-Enhancing Energy paradigm, J. Appl. Phycol. (2018) 1-10. https://doi.org/10.1007/s10811-018-1505-3

[12] X. Quero, N. Moreno, J.T. Umaña, A. Alastuey, E. Hernández, A. Lopez-Soler, F. Plana, Synthesis of zeolites from coal fly ash: an overview, Int. J. Coal Geol. 50 (2002) 413-423. https://doi.org/10.1016/S0166-5162(02)00124-6

[13] C. Colella, Natural zeolites in environmentally friendly processes and applications. Stud. Surf. Sci. Catal. 125 (1999) 641-655. https://doi.org/10.1016/S0167-2991(99)80270-5

[14] J. Hlavay, G.Y. Vigh, V. Olaszi, J. Inczedy, Investigations on natural Hungarian zeolite for ammonia removal, Water Res. 16 (1982) 417-420. https://doi.org/10.1016/0043-1354(82)90165-8

[15] M. Kithome, J.W. Paul, L.M. Lavkulich, A.A. Bomke, Kinetics of ammonium adsorption and desorption by the natural zeolite clinoptilolite, Soil Sci. Soc. Am. J. 62 (1998) 622-629. doi:10.2136/sssaj1998.03615995006200030011x

[16] C.-T. Kuo, Adsorbent-based Algal Cultivation System to Facilitate Integration of Algal Biofuel Production with Wastewater Treatment. PhD dissertation. Department of Agricultural and Biological Engineering, Graduate College, University of Illinois at Urbana-Champaign, 2017. http://hdl.handle.net/2142/97762

[17] M.R. Bilad, H.A. Arafat, I.F. Vankelecom, Membrane technology in microalgae cultivation and harvesting: a review, Biotechnol. Adv. 32 (2014) 1283-1300. https://doi.org/10.1016/j.biotechadv.2014.07.008

[18] F. Gao, Y.Y. Peng, C. Li, W. Cui, Z.H. Yang, G.M. Zeng, Coupled nutrient removal from secondary effluent and algal biomass production in membrane photobioreactor (MPBR): Effect of HRT and long-term operation, Chem. Eng. J. 335 (2018) 169-175. https://doi.org/10.1016/j.cej.2017.10.151

[19] Y. Wang, S. Liu, Z. Xu, T. Han, S. Chuan, T. Zhu, Ammonia removal from leachate solution using natural Chinese clinoptilolite, J. Hazard. Mater. 136 (2006) 735-740. https://doi.org/10.1016/j.jhazmat.2006.01.002

[20] L. Qin, Q. Shu, Z. Wang, C. Shang, S. Zhu, J. Xu, R. Li, L. Zhu, Z. Yuan, Cultivation of *Chlorella vulgaris* in dairy wastewater pretreated by UV irradiation and sodium hypochlorite, Appl. Biochem. Biotechnol. 172 (2014) 1121-1130. https://doi.org/10.1007/s12010-013-0576-5

[21] D.T. Newby, T.J. Mathews, R.C. Pate, M.H. Huesemann, T.W. Lane, B.D. Wahlen, S. Mandal, R.K. Engler, K.P. Feris, J.B. Shurin, Assessing the potential of polyculture to accelerate algal biofuel production, Algal Res. 19 (2016) 264-277. https://doi.org/10.1016/j.algal.2016.09.004

[22] M. Stockenreiter, F. Haupt, J. Seppälä, T. Tamminen, K. Spilling, Nutrient uptake and lipid yield in diverse microalgal communities grown in wastewater. Algal Res. 15 (2016) 77-82. https://doi.org/10.1016/j.algal.2016.02.013

[23] UTEX. Bold 1NV Medium Recipe. https://utex.org/products/bold-1nv-medium (accessed 1 May 2018).

[24] D.W. Gao, Q. Hu, C. Yao, N.Q. Ren, W.M. Wu, Integrated anaerobic fluidized-bed membrane bioreactor for domestic wastewater treatment, Chem. Eng. J. 240 (2014) 362-368. https://doi.org/10.1016/j.cej.2013.12.012

[25] The Florida Department of Environmental Protection (FDEP), 2017 Annual Drinking Water Quality Report. https://www.usf.edu/administrative-services/documents/drinking-water-quality-report-2017.pdf (accessed 1 September 2018).

[26] S. Morr, E. Cuartas, B. Alwattar, J.M. Lane, How much calcium is in your drinking water? A survey of calcium concentrations in bottled and tap water and their significance for medical treatment and drug administration, HSS J. 2 (2006) 130-135. https://doi.org/10.1007/s11420-006-9000-9

[27] S. Wang, Y. Peng, Natural zeolites as effective adsorbents in water and wastewater treatment, Chem. Eng. J. 156 (2010) 11-24. https://doi.org/10.1016/j.cej.2009.10.029

[28] G. Markou, O. Depraetere, D. Vandamme, K. Muylaert, Cultivation of *Chlorella vulgaris* and *Arthrospira platensis* with recovered phosphorus from wastewater by means of zeolite sorption, Int. J. Mol. Sci. 16 (2015) 4250-4264. https://doi.org/10.3390/ijms16024250

[29] M. Nieves, D. Voltolina, P. Piña, Growth and biomass production of *Tetraselmis suecica* and *Dunaliella tertiolecta* in a standard medium added with three products of zeolitic nature, Aquacult. Eng. 32 (2005) 403-410. https://doi.org/10.1016/j.aquaeng.2004.09.003

[30] J. González-Camejo, R. Barat, M.V. Ruano, A. Seco, J. Ferrer, Outdoor flat-panel membrane photobioreactor to treat the effluent of an anaerobic membrane bioreactor. Influence of operating, design, and environmental conditions, Wat. Sci. Tech. 78 (2018) 195-206. https://doi.org/10.2166/wst.2018.259

[31] L.L. Zhuang, H.Y. Hu, Y.H. Wu, T. Wang, T.Y. Zhang, A novel suspended-solid phase photobioreactor to improve biomass production and separation of microalgae, Bioresour. Technol. 153 (2014) 399-402. https://doi.org/10.1016/j.biortech.2013.12.035

[32] Y. Chisti, Biodiesel from microalgae, Biotechnol. Adv. 25 (2007) 294-306.

[33] A. Juneja, R.M. Ceballos, G.S. Murthy, Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: a review, Energies 6 (2013) 4607-4638. https://doi.org/10.3390/en6094607

[34] N.C. Boelee, H. Temmink, M. Janssen, C.J.N. Buisman, R.H. Wijffels, Nitrogen and phosphorus removal from municipal wastewater effluent using microalgal biofilms, Water Res. 45 (2011) 5925-5933. https://doi.org/10.1016/j.watres.2011.08.044

[35] C. Zamalloa, N. Boon, W. Verstraete, Decentralized two-stage sewage treatment by chemical–biological flocculation combined with microalgae biofilm for nutrient immobilization in a roof installed parallel plate reactor, Bioresour. Technol. 130 (2013) 152-160. https://doi.org/10.1016/j.biortech.2012.11.128

[36] M.L. Nguyen, Retention and subsequent nitrification of wastewater ammonium in natural New Zealand zeolites. In Proc., UNEP—For Life on Earth—Int. Regional Conf., Murdoch University, Perth, Australia, 1997.

[37] L.E. González, R.O. Cañizares, S. Baena, Efficiency of ammonia and phosphorus removal from a Colombian agroindustrial wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*, Bioresour. Technol. 60 (1997) 259-262. https://doi.org/10.1016/S0960-8524(97)00029-1

[38] R. Tao, V. Kinnunen, R. Praveenkumar, A.-M. Lakaniemi, J.A. Rintala, Comparison of *Scenedesmus acuminatus* and *Chlorella vulgaris* cultivation in liquid digestates from anaerobic digestion of pulp and paper industry and municipal wastewater treatment sludge, J. Appl. Phycol. 29 (2017) 2845-2856. https://doi.org/10.1007/s10811-017-1175-6

[39] J. Huisman, R.R. Jonker, C. Zonneveld, F.J. Weissing, Competition for light between phytoplankton species: experimental tests of mechanistic theory, Ecol. 80 (1999) 211-222. https://doi.org/10.1890/0012-9658(1999)080[0211:CFLBPS]2.0.CO;2

[40] R.C. Leitão, A.C. Van Haandel, G. Zeeman, G. Lettinga, The effects of operational and environmental variations on anaerobic wastewater treatment systems: a review, Bioresour. Technol. 97 (2006) 1105-1118. https://doi.org/10.1016/j.biortech.2004.12.007

[41] J.C. Goldman, P.G. Brewer, Effect of nitrogen source and growth rate on phytoplankton-mediated changes in alkalinity, Limnol. Oceanogr. 25 (1980) 352-357. https://doi.org/10.4319/lo.1980.25.2.0352

[42] J.C. Lee, J.S. Kim, I.J. Kang, M.H. Cho, P.K. Park, C.H. Lee, Potential and limitations of alum or zeolite addition to improve the performance of a submerged membrane bioreactor, Wat. Sci. Tech. 43 (2001) 59-66. https://doi.org/10.2166/wst.2001.0667

[43] J. Wanner, Activated sludge: bulking and foaming control. CRC Press, 2014.

[44] J.J. Cole, Interactions between bacteria and algae in aquatic ecosystems, Annu. Rev. Ecol. Syst. 13 (1982) 291-314. https://doi.org/10.1146/annurev.es.13.110182.001451

[45] M.B. Kasiri, H. Aleboyeh, A. Aleboyeh, Degradation of Acid Blue 74 using Fe-ZSM5 zeolite as a heterogeneous photo-Fenton catalyst, Appl. Catal. B. 84 (2008) 9-15. https://doi.org/10.1016/j.apcatb.2008.02.024

[46] W.D.P. Stewart, ed. Algal physiology and biochemistry. Vol. 10. Univ of California Press 1974.

[47] X. Li, H. Xu, Q. Wu, Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors, Biotechnol. Bioeng. 98 (2007) 764-771. https://doi.org/10.1002/bit.21489

[48] A. Viarengo, Biochemical effects of trace metals, Mar. Pollut. Bull. 16 (1985) 153-158. https://doi.org/10.1016/0025-326X(85)90006-2

# 7 General discussion and conclusions

## 7.1 General discussion

In this study, *Chlorella vulgaris* and *Scenedesmus acuminatus* were shown to grow in wastewaters with high ammonium concentration and turbidity and to efficiently remove nutrients and organic matter from the wastewaters (Chapter 3 and 4). Promisingly, the highest obtained microalgal biomass concentration of *S. acuminatus* in liquid digestates from pulp and paper industry was among one of the highest reported for microalgae in real wastewaters. Due to the differences observed in *S. acuminatus* growth in liquid digestates obtained at different AD conditions, sulfate was considered as one potential reason for the different nutrient removal efficiency and microalgal biomass concentration (Chapter 4). However, the effect of sulfate on nitrogen removal efficiency and microalgal growth was not significant when assessed in synthetic medium using factorial experimental design (Chapter 5). Thus, sulfate was not likely a significant factor for the observed robust microalgal growth and efficient nutrient removal in liquid digestates from pulp and paper industry. Apart from studying sulfate concentration, addition of a low concentration of zeolite to a membrane photobioreactor was shown to promote ammonium removal efficiency and microalgal growth (Chapter 6). The main findings from each study/chapter of this thesis are shown in Figure 7.1.

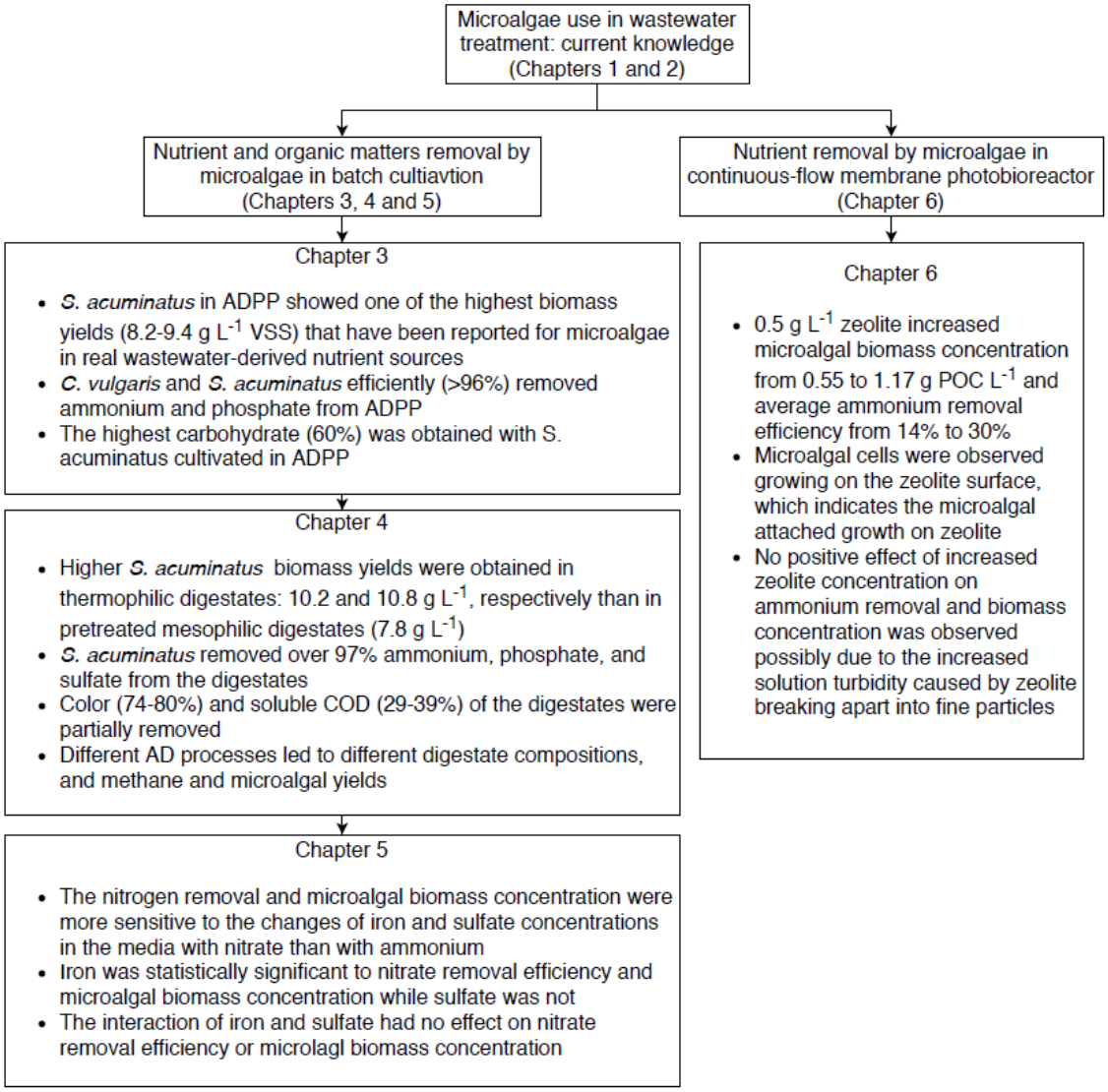



**Figure 7.1** Major results of each chapter in this thesis.

Both microalgal monocultures and a mixed culture were studied and the changes of the microalgal cell morphology or species composition were observed under the microscope as the microalgae needed to adapt to the changing cultivation conditions (Chapter 3–6). In practice, it is common that wastewater compositions vary widely due to operational and environmental variations (Leitão et al., 2006). In this study, *C. vulgaris* and *S. acuminatus* were able to grow in wastewaters with different composition and *S. acuminatus* enabled efficient nutrient removal from liquid digestates of pulp and paper industry. The fact that higher nutrient removal efficiency and microalgal biomass concentration was obtained with *S. acuminatus* than with *C. vulgaris* indicates that *S. acuminatus* may be more

resistant than *C. vulgaris* to the organic compounds (e.g. lignin and its derivatives) often present in ADPP (Murray et al. 2010). Thus, selection of the suitable microalgal species for the specific wastewater is important and can be considered as a first step in developing efficient microalgae-based wastewater treatment.

Dilution plays also an important role in wastewater treatment with microalgae because undiluted wastewater may not be suitable for microalgal growth due to e.g. high ammonium concentration and/or turbidity. However, high dilution ratios can result in low microalgal biomass production due to insufficient availability of nutrients. Dilution with tap water might not be advisable as it would consume clean water and increase treatment costs due to larger treatment process volume. Alternatively, it would be possible to mix two or more wastewaters together to modify wastewater composition and N/P ratios to reach suitable conditions for microalgal growth. N/P ratios ranging from e.g. 1 to 10 have been reported to promote microalgal growth depending on the species and cultivation conditions (Choi and Lee, 2015; Klausmeier et al., 2004; Xin et al., 2010). In this study, the different N/P ratios in ADMW ($N_{ammonium}/P_{phosphate}$: 410:1) and in mesophilic ADPP ($N_{ammonium}/P_{phosphate}$: 14.6:1) might be one of the reasons for the higher microalgal biomass concentrations obtained in mesophilic ADPP (Chapter 3). In practical applications, it is also possible to recycle the treated wastewater (after cultivation) back to the treatment process if dilution is needed.

The obtained results show that different AD processes resulted in different digestate composition, and different nutrient and organic matter removal by microalgal cultivation from the resulting digestate (Chapter 4). It was also shown that the highest methane production and microalgal biomass concentration can be obtained in the same integrated AD and microalgal cultivation system. This is a promising discovery to promote pulp and paper industry microalgal-based biorefinery applications as AD treatment of wastewater and/or sludge from pulp and paper mills has been studied and applied in Finland (Kamali et al., 2016; Kinnunen et al., 2015; Kokko et al., 2018; Liikanen, 2016). In addition to treating digestate, microalgal cultivation could utilize $CO_2$ generated during AD to further integrate microalgae and AD in wastewater treatment as shown in Figure 7.2. The liquid fraction after microalgal cultivation could be recycled to activated-sludge process for remove residual nutrients and organic matter (e.g. COD and DOC). The produced and harvested microalgal biomass is a potential feedstock for e.g. fertilizer and bioenergy production.

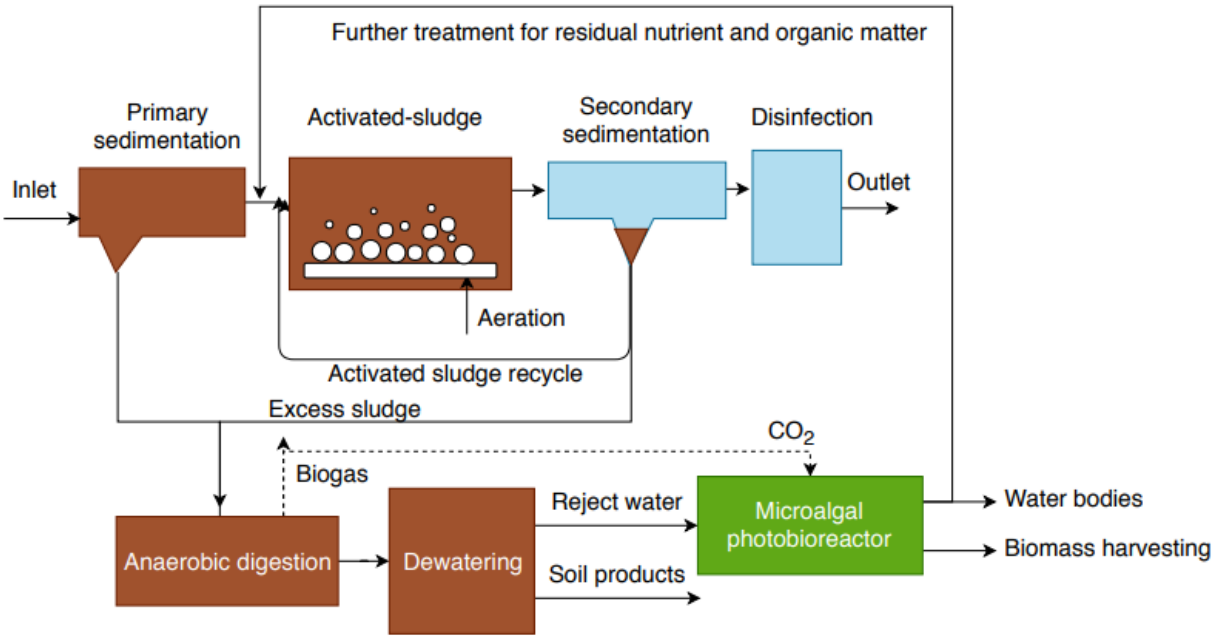



**Figure 7.2** Schematic representation on possible ways to integrate microalgal cultivation to anaerobic digestion. Solid lines represent the liquid flow (wastewaters) and dashed lines gas flow (biogas or $CO_2$).

The findings on the effects of sulfate and zeolite addition on nitrogen removal and algal growth implicate the potential to affect and optimize the nutrient removal and microalgal growth in various ways. Some minor effects of sulfate on nitrogen removal efficiency and microalgal growth were found and the effects varied depending on the nitrogen source in the medium (Chapter 5). Thus, sulfate in wastewaters would not be considered as a main contributor for varied nitrogen removal efficiency or microalgal growth, but the presence of small amount of sulfate is important for microalgal growth and nutrient removal. In the membrane photobioreactor study, the fine particles of zeolite released to the culture due to shear stress can be considered as zeolite loss as they could flow away as permeate or during sampling. Although natural zeolite is considered as a cheap resource, high amount of zeolite would be needed in large-scale applications due to large wastewater volume and possible zeolite loss. Different zeolite concentrations resulted in varied treatment efficiency and solution turbidity increased with increasing zeolite concentration and residence time in the system. Thus, it is important to understand the effects of zeolite concentration on wastewater efficiency with time and the zeolite loss rate to decide when to compensate the zeolite loss.

## 7.2 Recommendations for future research

Although the use of *S. acuminatus* for treating liquid digestates from pulp and paper industry seems to be a promising application area for microalgae based on this study, the process needs further optimization before commercialization. For example, the optimal conditions by integrating of AD and microalgal cultivation need to be analyzed in larger scale with different operation modes (batch, semi-batch, semi-continuous, and continuous) to design the reactor and concept configuration and operation strategy with efficient pollutants removal and biomass production. In practice, the turbidity of reject waters may be higher than that used in this study due to different filtration units. Thus, pretreatment such as dilution should be considered as varied compositions result in different treatment efficiency and biomass production. In addition, *S. acuminatus* can also be studied for treating other wastewaters generated from pulp and paper industry.

In microalgal cultivation, it is often challenging to quantitatively determine the assimilated and precipitated fractions of the removed phosphorus and these have typically not been differentiated. Polomski et al. (2009) and Sindelar et al. (2015) estimated theoretically the amount of precipitated phosphorus using a chemical equilibrium software application to calculate e.g. speciation, solubility, sorption of solid and dissolved phases of minerals in aqueous systems. Furthermore, Sindelar et al. (2015) determined the amount of phosphorus assimilated by microalgae by using inductively coupled plasma-atomic emission spectroscopy after digesting the microalgal biomass. However, accuracy of this method may be limited, as it is hard to separate the precipitated phosphorus from the microalgal biomass. One possible way to verify the quantification of precipitated and cellular phosphorus is to calculate the total amount of removed phosphorus based on the initial and final phosphorus concentrations of the wastewater. The methods can be considered reliable when the total removed phosphorus is equal/close to the sum of the estimated precipitated and biomass-bound phosphorus. Thus, further studies are needed for these quantification methods to provide more in-depth understanding of the pathways occurring during phosphorus removal by microalgae.

Microalgae use in wastewater treatment can be carried out in batch and/or continuous modes as well as open and closed cultivation systems. The system to be used in specific case depends on e.g. the amount and generated modes (e.g. batch or continuous) of wastewaters, available land areas, local climate conditions, post treatment systems and compounds that need to be removed. In addition, the optimizations of process parameters (e.g. temperature, light intensity, and HRT and SRT of continuous system) are also essential for efficient treatment. For example, in this study, the ammonium removal was not efficient in the membrane photobioreactor experiment, because the initial ammonium concentration was too high to be utilized by microalgae within the used HRT. In the future work, longer HRT should be used to enable more efficient nutrient removal.

## 7.3 Conclusions

In this study, *Chlorella vulgaris* and *Scenedesmus acuminatus* were able to grow in liquid digestates resulting from anaerobic digestion of biosludge from a municipal wastewater treatment plant (ADMW) and a pulp and paper mill wastewater treatment plant (ADPP). *C. vulgaris* and *S. acuminatus* removed ammonium efficiently (>97%) from ADPP, while the final ammonium removal efficiencies from ADMW with *C. vulgaris* and *S. acuminatus* were 24 and 44%, respectively. Both microalgae could efficiently remove phosphate (>96%) from the liquid digestates. Color (74–80%) and soluble COD (27–39%) of ADMW and ADPP were removed to a certain degree.

*S. acuminatus* cultivation in ADPP resulted in one of the highest biomass concentrations (7.8–10.8 g $L^{-1}$ VSS) that has been reported for microalgae in real wastewaters. In addition, higher growth of *S. acuminatus* was obtained in the undiluted ADPP than in the diluted ones. Different AD processes of biosludge from pulp and paper industry resulted in different digestate compositions (e.g. turbidity, ammonium concentration, and soluble COD), methane yields, and microalgal growth. Higher *S. acuminatus* biomass concentrations were obtained in thermophilic digestates (10.2–10.8 g $L^{-1}$) than in pretreated mesophilic digestate (7.8±0.3 g $L^{-1}$), likely due to differences in concentration of minor nutrients. Importantly, the highest microalgal biomass and methane yields in the pretreated thermophilic digestates indicates that the highest methane production and microalgal biomass yields can be obtained in the same integrated anaerobic digestion and microalgal cultivation system.

Nitrogen removal efficiency and microalgal biomass concentration were more sensitive to the changes in iron and sulfate concentrations in the media with nitrate than with ammonium, probably due to different assimilation mechanisms used by microalgae for the two nitrogen sources. In the present study, the highest microalgal biomass concentration was obtained using 1.0 mg $L^{-1}$ iron and 35.8 mg $L^{-1}$ sulfate-sulfur in the medium with nitrate as nitrogen source. Iron concentration had statistically significant impact on ammonium removal and microalgal growth while sulfate concentration had no impact. However, the interaction between iron and sulfate did not affect the ammonium removal efficiency and microalgal growth.

Addition of low concentration of zeolite (0.5 g $L^{-1}$) to a continuous-flow membrane photobioreactor increased average ammonium removal efficiency (14 to 30%) and microalgal biomass concentration (0.50 to 1.17 g POC $L^{-1}$). This was likely because zeolite provided a habitat for biofilm-based growth and zeolite adsorption of ammonium resulted in higher availability of ammonium for microalgal growth on the zeolite surface. Increase in zeolite concentration (from 0.5 to 1 and 5 g $L^{-1}$) did not

enhance ammonium removal efficiency or biomass concentration. This was likely due to the increased solution turbidity caused by breaking apart of added zeolite particles into finer particles, which reduced light availability.

To sum up, this study demonstrated the possibility to use microalgae in wastewater treatment with efficient nutrient removal and partial organic matter removal. However, it is important to select suitable microalgal species for the specific wastewater to enable efficient nutrient removal and high microalgal biomass production.

## References


Bohutskyi, P., Kligerman, D.C., Byers, N., Nasr, L.K., Cua, C., Chow, S., Su, C., Tang, Y., Betenbaugh, M.J., Bouwer, E.J., 2016. Effects of inoculum size, light intensity, and dose of anaerobic digestion centrate on growth and productivity of *Chlorella* and *Scenedesmus* microalgae and their poly-culture in primary and secondary wastewater. Algal Research, 19, 278-290.

Choi, H.J., Lee, S.M., 2015. Effect of the N/P ratio on biomass productivity and nutrient removal from municipal wastewater. Bioprocess and Biosystems Engineering, 38(4), 761-766.

Kamali, M., Gameiro, T., Costa, M.E.V., Capela, I., 2016. Anaerobic digestion of pulp and paper mill wastes–An overview of the developments and improvement opportunities. Chemical Engineering Journal, 298, 162-182.

Kinnunen, V., Ylä-Outinen, A., Rintala, J., 2015. Mesophilic anaerobic digestion of pulp and paper industry biosludge–long-term reactor performance and effects of thermal pretreatment. Water Research, 87, 105-111.

Klausmeier, C.A., Litchman, E., Daufresne, T., Levin, S.A., 2004. Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. Nature, 429(6988), 171.

Kokko, M., Koskue, V., Rintala, J., 2018. Anaerobic digestion of 30− 100-year-old boreal lake sedimented fibre from the pulp industry: Extrapolating methane production potential to a practical scale. Water Research, 133, 218-226.

Leitão, R.C., Van Haandel, A.C., Zeeman, G., Lettinga, G., 2006. The effects of operational and environmental variations on anaerobic wastewater treatment systems: a review. Bioresource Technology, 97(9), 1105-1118.

Liikanen, J., 2016, December 7. Biofuel from Äänekoski. Paper and Timber Journal. Retrieved from: http://www.paperijapuu.fi/biofuel-from-aanekoski/.

Murray, D., Jefferson, B., Jarvis, P., Parsons, S.A., 2010. Inhibition of three algae species using chemicals released from barley straw. Environmental Technology, 31(4), 455-466.

Polomski, R.F., Taylor, M.D., Bielenberg, D.G., Bridges, W.C., Klaine, S.J., Whitwell, T., 2009. Nitrogen and phosphorus remediation by three floating aquatic macrophytes in greenhouse-based laboratory-scale subsurface constructed wetlands. Water, Air, and Soil Pollution, 197(1-4), 223-232.

Sindelar, H.R., Yap, J.N., Boyer, T.H., Brown, M.T., 2015. Algae scrubbers for phosphorus removal in impaired waters. Ecological Engineering, 85, 144-158.

Wang, Z., Zhao, Y., Ge, Z., Zhang, H., Sun, S., 2016. Selection of microalgae for simultaneous biogas upgrading and biogas slurry nutrient reduction under various photoperiods. Journal of Chemical Technology & Biotechnology, 91(7), 1982-1989.

Watanabe, T., Masaki, K., Iwashita, K., Fujii, T., Iefuji, H., 2009. Treatment and phosphorus removal from high-concentration organic wastewater by the yeast *Hansenula anomala* J224 PAWA. Bioresource Technology, 100(5), 1781-1785.

Xin, L., Hong-ying, H., Ke, G., Ying-xue, S., 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresource Technology, 101(14), 5494-5500.

Zuliani, L., Frison, N., Jelic, A., Fatone, F., Bolzonella, D., Ballottari, M., 2016. Microalgae cultivation on anaerobic digestate of municipal wastewater, sewage sludge and agro-waste. International Journal of Molecular Sciences, 17(10), 1692.

# Appendixes: supporting information for Chapters 4, 5, and 6

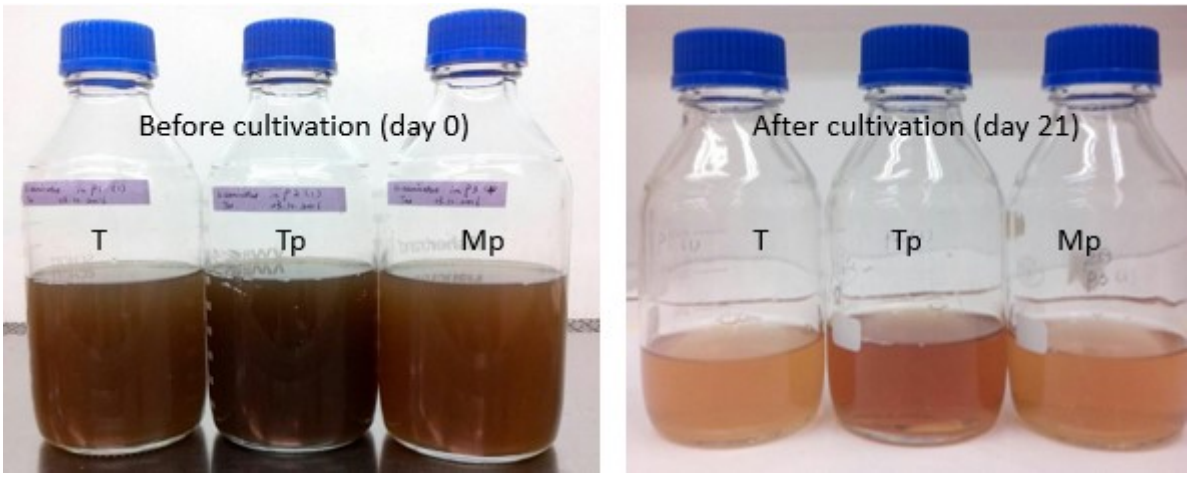



**Figure S4.1** The photos of liquid digestates from the pulp and paper wastewater treatment plant biosludge, anaerobically treated under thermophilic conditions (55 °C) without pretreatment (T), with pretreatment (121 °C) for 10 min (Tp), and under mesophilic conditions (35 °C) with pretreatment (121 °C) for 10 min (Mp) before (day 0) and after cultivation (day 21).

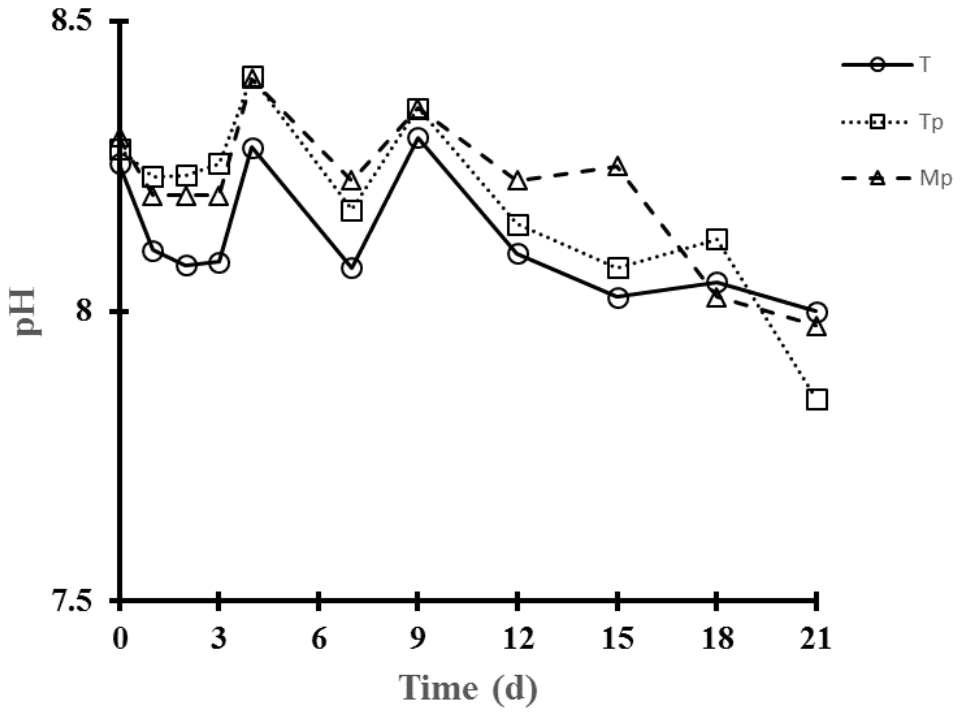



**Figure S4.2** pH evolution during the cultivation of *Scenedesmus acuminatus* in the liquid digestates from the pulp and paper wastewater treatment plant biosludge, anaerobically treated under thermophilic conditions (55 °C) without pretreatment (T), with pretreatment (121 °C) for 10 min (Tp), and under mesophilic conditions (35 °C) with pretreatment (121 °C) for 10 min (Mp).

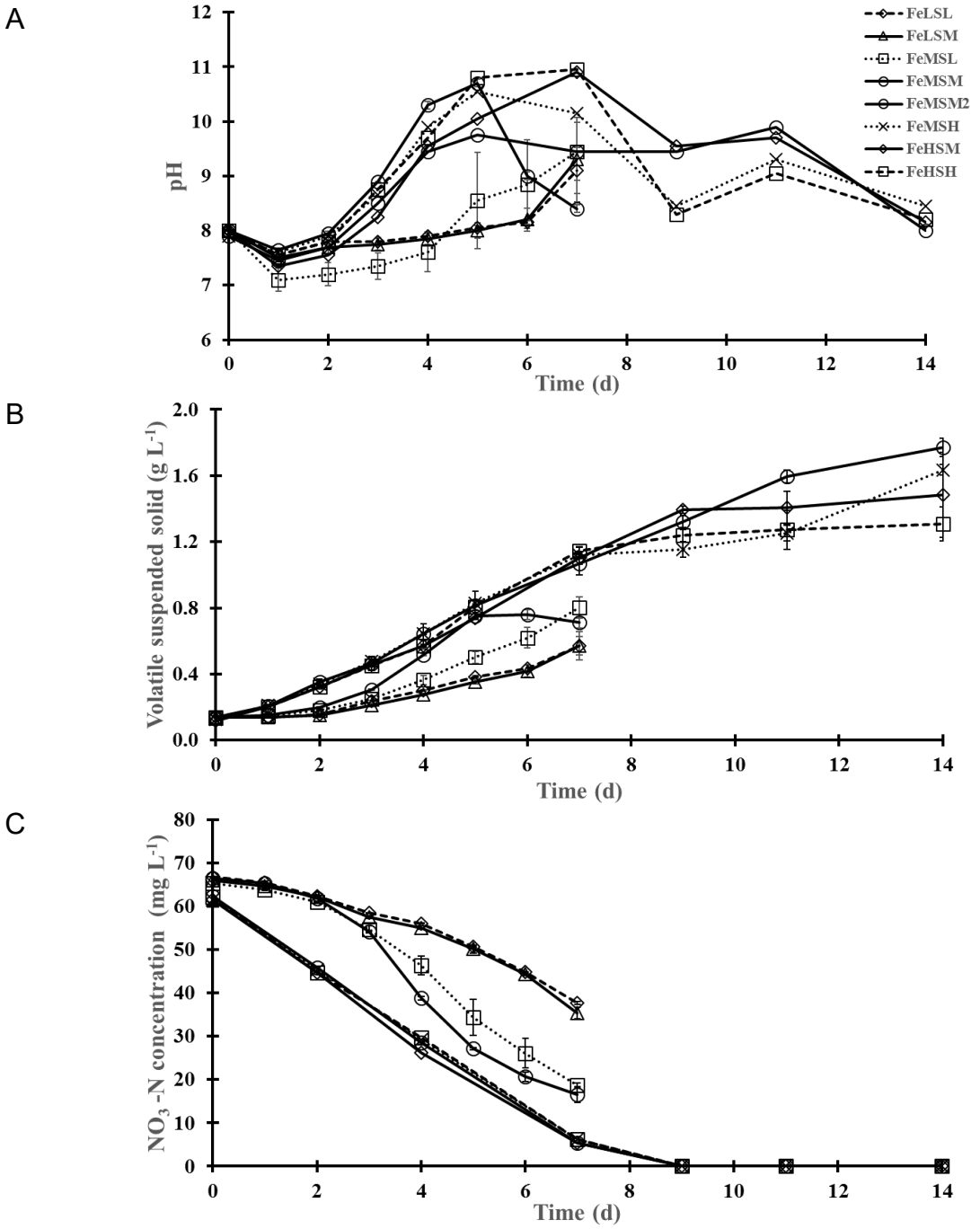


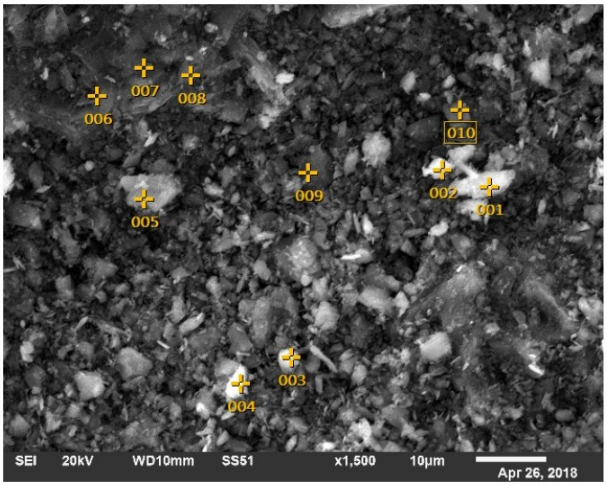

**Figure S5.1** pH (A), microalgal biomass concentration (as g VSS $L^{-1}$) (B) and nitrate-N (C) during the cultivation of *Scenedesmus acuminatus* in the modified N-8 media. The results of pH are presented as the means of n = 2 (2 cultivations, 1 measurement from each); error bars represent standard error. The results of VSS and nitrate are presented as the means of n = 4 (2 cultivations, 2 measurements from each); error bars represent standard deviation.

**Table S5.1** Most of the possible regression results of final microalgal biomass concentration in the $NO_3$ assay to select the best model according to p-value of overall model and the coefficient of determination (adjusted $R^2$)

| Variable(s) in model | $R^2$ | Adjusted $R^2$ | P-value | P-value <0.05 |
|---|---|---|---|---|
| Iron | 0.4932 | 0.457 | $2.418 \times 10^{-3}$ | Iron |
| Sulfur | 0.3443 | 0.2975 | $1.688 \times 10^{-2}$ | Sulfur |
| Iron, sulfur | 0.5673 | 0.5007 | $4.319 \times 10^{-3}$ | Iron |
| Iron, sulfur, iron*sulfur | 0.6045 | 0.5057 | $9.116 \times 10^{-3}$ | Iron |
| Iron, sulfur, iron$^2$ | 0.7688 | 0.711 | $4.007 \times 10^{-4}$ | Iron, iron$^2$ |
| Iron, sulfur, iron*sulfur, iron$^2$ | 0.7754 | 0.6937 | $1.426 \times 10^{-3}$ | Iron, iron$^2$ |
| Iron, sulfur, sulfur$^2$ | 0.5685 | 0.4606 | $1.502 \times 10^{-2}$ | Iron |
| Iron, sulfur, iron*sulfur, iron$^2$, sulfur$^2$ | 0.7924 | 0.6885 | $3.406 \times 10^{-3}$ | Iron, iron$^2$ |
| Iron, sulfur, iron*sulfur,sulfur$^2$ | 0.6133 | 0.4727 | $2.35 \times 10^{-2}$ | Iron |
| Sulfur, sulfur$^2$ | 0.3455 | 0.2448 | $6.358 \times 10^{-2}$ | |
| Iron, iron$^2$ | 0.6948 | 0.6479 | $4.464 \times 10^{-4}$ | Iron, iron$^2$ |
| Iron, iron$^2$, sulfur$^2$ | 0.7304 | 0.6631 | $9.864 \times 10^{-4}$ | Iron, iron$^2$ |
| Iron, sulfur, iron$^2$, sulfur$^2$ | 0.77 | 0.6864 | $1.614 \times 10^{-3}$ | Iron, iron$^2$ |

**Table S5.2** Most of the possible regression results of nitrate removal efficiency in the $NO_3$ assay to select the best model according to p-value of overall model and coefficient of determination (adjusted $R^2$)

| Variable(s) in model | $R^2$ | Adjusted $R^2$ | P-value | P-value <0.05 |
|---|---|---|---|---|
| Iron | 0.61 | 0.5821 | $3.549 \times 10^{-4}$ | Iron |
| Sulfur | 0.1808 | 0.1223 | $1.006 \times 10^{-1}$ | |
| Iron, sulfur | 0.6116 | 0.5518 | $2.141 \times 10^{-3}$ | Iron |
| Iron, sulfur, iron*sulfur | 0.7261 | 0.6576 | $1.083 \times 10^{-3}$ | Iron, iron*sulfur |
| Iorn, sulfur, iron$^2$ | 0.8567 | 0.8209 | $2.373 \times 10^{-5}$ | Iron, iron$^2$ |
| Iron, sulfur, iron*sulfur, iron$^2$ | 0.8609 | 0.8103 | $1.114 \times 10^{-4}$ | Iron, iron$^2$ |
| Iron, sulfur, sulfur$^2$ | 0.6394 | 0.5493 | $5.356 \times 10^{-3}$ | Iron |
| Iron, sulfur, iron*sulfur, iron$^2$, sulfur$^2$ | 0.8903 | 0.8355 | $1.612 \times 10^{-4}$ | Iron, iron$^2$ |
| Iron, sulfur, iron*sulfur,sulfur$^2$ | 0.7273 | 0.6282 | $3.936 \times 10^{-3}$ | Iron |
| Sulfur, sulfur$^2$ | 0.2086 | 0.08688 | $2.185 \times 10^{-1}$ | |
| Iron, iron$^2$ | 0.8551 | 0.8329 | $3.516 \times 10^{-6}$ | Iron, iron$^2$ |
| Iron, iron$^2$, sulfur$^2$ | 0.8607 | 0.8259 | $2.011 \times 10^{-5}$ | |
| Iron, sulfur, iron$^2$, sulfur$^2$ | 0.8846 | 0.8426 | $4.081 \times 10^{-5}$ | Iron, iron$^2$ |

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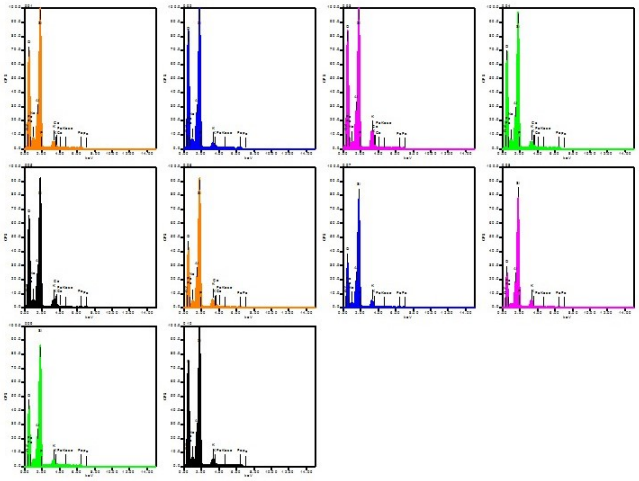

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Live time : 30.00 sec.
Real Time : 30.94 sec.
DeadTime : 4.00 %
Count Rate : 2137.00 CPS

| | P | Fe | K | O | C | N | Na | Al | Si | Ca | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 001 | 0.04 | 0.65 | 1.63 | 52.34 | 10.34 | 0.71 | 2.70 | 5.21 | 25.74 | 0.62 | |
| 002 | 0.01 | 0.58 | 1.50 | 55.25 | 9.05 | nd | 2.24 | 5.03 | 26.34 | | |
| 003 | 0.04 | 0.56 | 4.54 | 54.87 | 9.42 | 0.06 | 1.61 | 5.09 | 23.29 | 0.52 | |
| 004 | 0.04 | 0.74 | 2.14 | 53.49 | 6.94 | nd | 2.46 | 5.43 | 28.09 | 0.67 | |
| 005 | 0.09 | 0.35 | 2.33 | 53.12 | 7.41 | nd | 2.43 | 5.64 | 27.97 | 0.66 | |
| 006 | nd | 0.20 | 2.89 | 48.03 | 5.87 | 0.49 | 2.29 | 6.21 | 33.45 | 0.56 | |
| 007 | nd | 0.26 | 3.07 | 46.34 | 7.10 | nd | 2.06 | 6.23 | 34.95 | | |
| 008 | 0.25 | 0.43 | 4.29 | 43.49 | 4.72 | nd | | 7.03 | 39.79 | | |
| 009 | nd | 0.78 | 2.75 | 50.32 | 7.06 | nd | | 5.79 | 33.30 | | |
| 010 | 0.03 | 0.31 | 2.10 | 53.99 | 7.67 | 0.68 | 2.44 | 5.11 | 27.68 | | |
| Average | 0.07 | 0.49 | 2.73 | 51.12 | 7.56 | 0.48 | 2.28 | 5.68 | 30.06 | 0.61 | |
| Standard deviation | | 0.08 | 0.20 | 1.03 | 3.97 | 1.67 | 0.30 | 0.33 | 0.65 | 5.09 | 0.07 |

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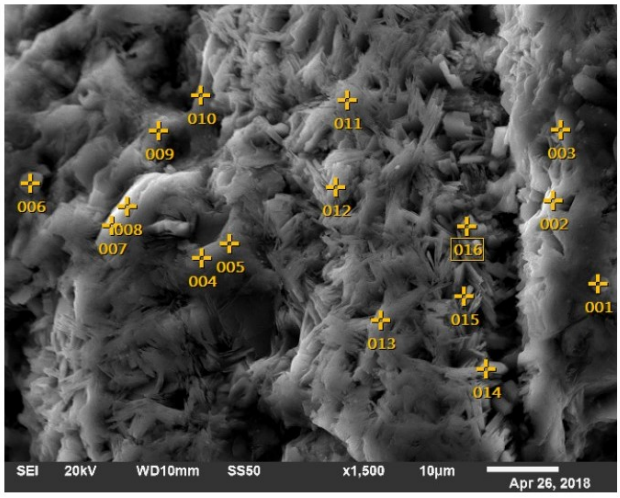
**Figure S6.1** Element proportion of raw zeolite in weight obtained from EDX analysis

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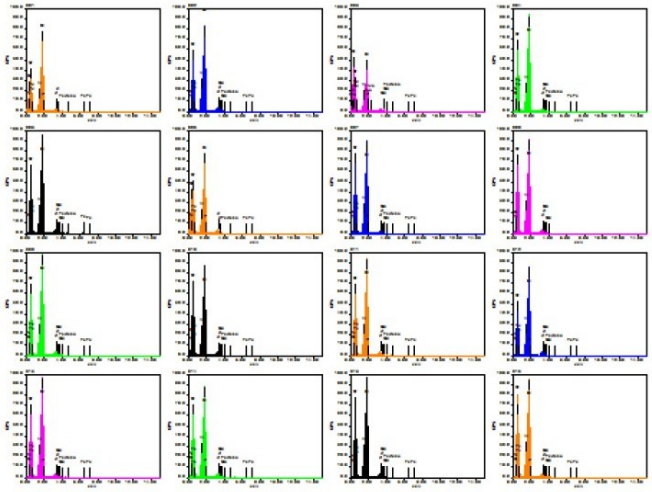

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Real Time : 30.94 sec.
DeadTime : 3.00 %
Count Rate : 2055.00 CPS

| | P | Fe | K | O | C | N | Al | Si | Ca | Mo |
|---|---|---|---|---|---|---|---|---|---|---|
| 001 | 0.14 | 0.31 | 0.73 | 37.85 | 35.63 | 10.21 | 1.96 | 13.17 | | |
| 002 | | 0.14 | 1.54 | 50.96 | 18.37 | 0.35 | 4.71 | 23.22 | 0.70 | |
| 003 | 0.27 | 0.03 | | 29.48 | 47.24 | 13.65 | 1.41 | 6.81 | 0.45 | 0.66 |
| 004 | | 0.50 | 1.50 | 54.14 | 11.17 | 1.22 | 4.26 | 26.59 | 0.61 | |
| 005 | | 1.42 | 1.68 | 53.37 | 11.13 | | 4.41 | 27.48 | 0.51 | |
| 006 | 0.18 | 0.26 | 0.67 | 37.51 | 40.03 | 9.47 | 1.93 | 9.95 | | |
| 007 | 0.05 | 0.10 | 1.05 | 54.34 | 17.49 | 1.33 | 3.81 | 21.27 | 0.55 | |
| 008 | | | 1.26 | 54.30 | 17.75 | nd | 4.41 | 21.74 | 0.53 | |
| 009 | | 0.23 | 1.56 | 53.51 | 12.28 | 1.11 | 4.64 | 26.01 | 0.65 | |
| 010 | 0.22 | 0.32 | 1.01 | 50.33 | 22.31 | 5.39 | 3.14 | 16.79 | 0.48 | |
| 011 | 0.05 | 0.01 | 1.40 | 54.83 | 11.60 | 0.03 | 4.99 | 26.44 | 0.66 | |
| 012 | 0.07 | 0.22 | 2.12 | 50.79 | 11.10 | 0.81 | 5.70 | 28.43 | 0.77 | |

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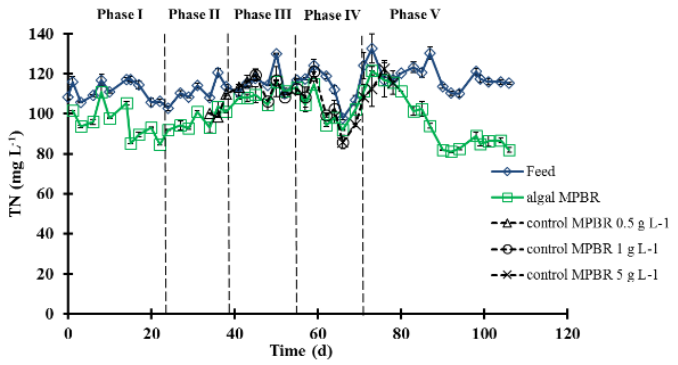
**Figure S6.2** Element proportion of used zeolite from the algal MPBR on day 108 in weight obtained from EDX analysis

A

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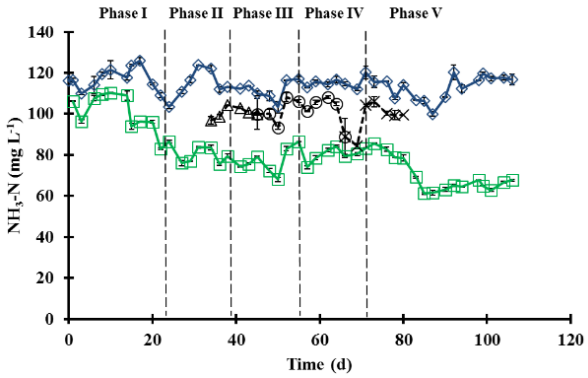


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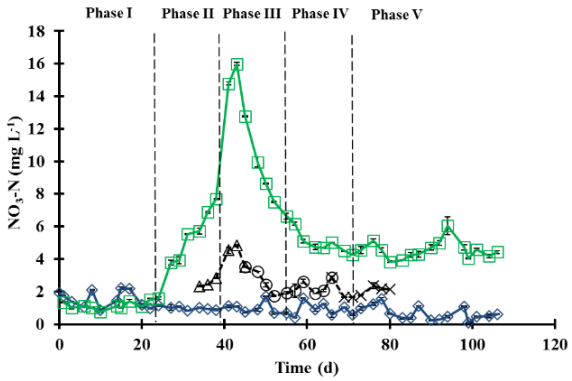


D

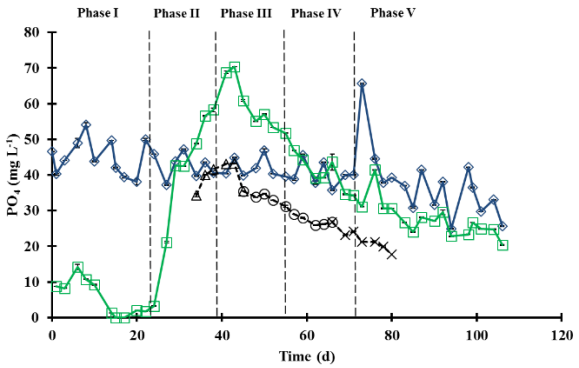


**Figure S6.3** The total nitrogen (A), ammonium-nitrogen (B), nitrate-nitrogen (C) and the total phosphate (D) concentrations of the feed and the permeate during the operation of membrane photobioreactors (the algal MPBR and the control MPBR). The results of total nitrogen, ammonium-nitrogen, nitrate-nitrogen, and total phosphate are presented as the means of n = 2; error bars represent standard deviation.