ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

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Ph.D. THESIS

Hande ERMİŞ

Department of Environmental Engineering

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ANAEROBİK ÇÜRÜTÜCÜ ÇIKIŞ SUYUNDA BÜYÜTÜLEN MİKROALGLERDEN DEĞERLİ ÜRÜN GERİ KAZANIMI

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Hande Ermis, a Ph.D. student of İTU Graduate School of Science Engineering and Technology student ID 501142804, successfully defended the thesis/dissertation entitled "MICROALGAE GROWTH IN ANAEROBIC DIGESTATE FOR HIGH-VALUE PRODUCT RECOVERY", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

"Living is no laughing matter,
you must live with great seriousness" said Nazım HIKMET and continued:
"Like a squirrel, for example,
I mean without looking for something beyond and above living,
I mean living must be your whole occupation.
Or else in a laboratory,
in your white coat and safety glasses,
you can die for people,
even for people whose faces you've never seen,
even though you know living,
is the most real, most beautiful thing."

This was a long journey, hence there will be many thanks for those holding my hand during this process. I would like to start by saying a heartfelt thank you to my dear supervisor Assoc. Dr. Mahmut ALTINBAS, to whom I could not express my gratitude enough. He was not only a supervisor, but also a mentor, a friend, a guide. He was the light at the end of a long, at times dark, tunnel. He was the one who told me to never give up every day, made me believe in myself, made me laugh when I cried of despair, and he was always there for me in every step of this path. I would also like to thank jury members of my thesis, Prof. Süleyman OVEZ and Assoc. Dr. Turgay CAKMAK, for their contributions and their endless support.

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Finally, I would like to thank to my family: Dear grandpas, thank you for teaching me to smile in every situation and to enjoy life: you both are my light, my angels, my protectors - until we meet again. Dear grandma, thank you for your love and having so much faith in me, for calling me everyday, for your prayers. Dear sister, thank you for not only being a role model for me but for all young women who you have been teaching, supervising, and helping out. You are my everything. Dear Dad, thank you for always making me feel that possibilities are endless for me no matter what. I never walked this earth alone because you are here. And dear mom, thank you so much for helping me to become the person I am today, and for being my biggest fan. I am who I am today because of you. And for this, I am forever grateful.

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Now, I am very much ready for the new chapter of my life, with gratitude and excitement.

The best is yet to come…

August 2020 Hande ERMİŞ

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ABBREVIATIONS

AD	**:** Anaerobic Digestion
ALD	**:** Anaerobic Liquid Digestate
Chl a	**:** Chlorophyll a
COD	**:** Chemical Oxygen Demand
DHA	**:** Docosahexaenoic acid
DM	**:** Dry Matter
FA	**:** Fatty acid
GC	**:** Gas Chromatography
ICP	**:** Inductively Coupled Plasma
MUFA	**:** Monounsaturated fatty acids
OD	**:** Optical Density
PCA	**:** Principal component analysis
PUFA	**:** Polyunsaturated fatty acids
ROS	**:** Reactive Oxygen Species
SFA	**:** Saturated Fatty Acid
TAG	**:** Triacylglyceride
TAN	**:** Total Ammonia Nitrogen
TKN	**:** Total Kjeldahl Nitrogen
TP	**:** Total Phosphorus
TSS	**:** Total Suspended Solid
VSS	**:** Volatile Suspended Solid

SYMBOLS

C/N	**:** Carbon to nitrogen ratio
k	**:** Reaction rate coefficient
K_a	**:** Dissociation constant for ammonium
K_m	**:** Half saturation constant
N:P	**:** Nitrogen to phosphorus ratio
R_i	**:** Removal rate
R_{xi}	**:** Specific rate of substrate removal
t_d	**:** Doubling time
Y_N	**:** Yield coefficient (Y_P) for nitrogen
Y_P	**:** Yield coefficient (Y_P) for phosphorus
μ	**:** Specific growth rate

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MICROALGAE GROWTH IN ANAEROBIC DIGESTATE FOR HIGH-VALUE PRODUCT RECOVERY

SUMMARY

As a result of diminished fossil fuel reserves and increased prices of petrochemical fuels, and the simultaneous result of fossil fuels on extreme greenhouse gas emissions leading global warming, the world is searching for an alternative renewable energy sources. Renewable energy sources such as solar, wind, hydro, geothermal and biomass have been successfully developed but research shows that biomass energy has the highest capability among other renewable sources. Microalgae has the highest attention in the field of bioenergy by not requiring arable land, their higher productivity and higher biofuel yield compared to terrestrial plants. Not only for bioenergy aspect, bu also for the environmental aspect, microalgae has too many advantages such as mitigation of CO_2 through photosynthesis and treatment of wastewater.

Anaerobic digestion is another and the most conventional methods used to generate energy from renewable sources. However, untreated anaerobic liquid digestate may cause eutrophication if directly discharges into the water sources due to its high nutrient content. Cultivating microalgae in digested effluents offers significant advantages in terms of wastewater treatment, the production of valuable biomass which can be further valorized, and a major decrease in the upstream cost of the process. Microalgae can assimilate nutrients especially nitrogen and phosphorous from wastewater for their growth and produce valuable biomass. Digestates include all essential macro/micro nutrients and can be recovered as cultivation media for microalgal biomass production. However, some of the main physicochemical characteristics of the digestates, such as high content of inhibitory compounds, turbidity, and colored dissolved compounds might negatively influence microalgal growth, and therefore they need to be adjusted by using one or a combination of pretreating methods (e.g., dilution, solid/ liquid separation, filtration, etc.) in order to provide suitable medium for cell growth.

The main objective of this thesis was to investigate the applicability of recovery of nutrients from digestate with mixed microalgae and observe positive effect on algal biomass resulting in enhanced high value product content. High-value products such as pigments, proteins, lipids, and carbohydrates that are obtained from microalgae grown in digestate can used as fuel, fertilizer and animal feed contributing to a substantial saving in the overall cost of microalgae biomass production. In this scope, the Anaerobic Liquid Digestate (ALD) obtained from real full scale plant to demonstrate and to show the applicability of succesful growth on digestate, instead of treating with advanced treatment methods and to recover high value products from the wild type algal biomass isolated from near-by water bodies in ITU to examine its dominancy change and positive effect. To promote the sustainability of microalgae–bacteria-based systems that treat wastewater, the examination of multiple products in the form of high value products and biomass were detailly monitored.

In the first part of the study, batch experiments were carried out to investigate the biokinetic coefficients for nutrient removal of mixed microalgae grown on anaerobic

liquid digestate by Michaelis–Menten rate expression. The initial NH_3-N concentration was varied between 18.6–87.1 mg L^{-1} while initial PO_4-P concentration was between 1.85–6.88 mg L^{-1}, which corresponds to 2%, 5%, 7% and 10% dilution ratio of anaerobic digestate. According to the yield results (mg chl a mg^{-1} nutrient), mixed microalgae uptake 10 times more nitrogen than phosphorus. Biokinetic coefficients were determined as k_N = 2.48 mg NH_3-N mg^{-1} chl a d^{-1}, Km_N = 29.3 mg L^{-1}, Y_N = 0.45 mg chl a mg^{-1} NH_3-N for nitrogen; and k_P = 0.21 mg PO4-P mg^{-1} chl a d^{-1}, Km_P = 2.94 mg L^{-1}, Y_P = 5.03 mg chl a mg^{-1} PO_4-P for phosphorus. The highest chlorophyll production (39 mg L^{-1} or 3.31 mg L^{-1} d^{-1}) was observed at the highest dilution ratio of 10%. Moreover, the highest dilution resulted in highest biomass (1.25 g L^{-1}) despite of dark, high ammonicial and particulate rich wastewater.

In the second stage of the study, mixed microalgal culture dominated by Chlorella vulgaris and Scenedesmus armatus were grown in Anaerobic Liquid Digestate (ALD) under different NaCl concentrations ranging between 0 to 100 mM. Highest lipid and carbohydrate amount were observed as 38% and 36%, respectively, when the salinity was 50 mM NaCl. However, the protein content was drastically decreased to 13% with increased NaCl concentration. Furthermore, the algal biomass was subsequently decreased along with total chlorophyll amount with increased NaCl concentration. Algal species showed diverse response to salinity stress and demonstrated a cost-effective approach towards the cultivation of mixed microalgae within digestate and provided insight that ALD and/or other wastewaters can be diluted with seawater instead of tap water in the future studies. This study could be further converted into biofuels due to high lipid increase along with the digestate treatment, which will help to valorize ALD and bring into economy.

In the third stage of the study, FeSO4 supplementation ranging from 0 to 4.5 mM, and MgSO4 supplementation ranging from 0 to 5.1 mM were investigated to observe the effect on the population dynamics, biochemical composition and fatty acid content of mixed microalgae grown in Anaerobic Liquid Digestate (ALD). Overall, 3.1 mM FeSO4 addition into ALD increased the total protein content 60% and led to highest biomass (1.56 g L^{-1}) and chlorophyll-a amount (18.7 mg L-1) produced. Meanwhile, 0.4 mM MgSO4 addition increased the total carotenoid amount 2.2 folds and slightly increased the biomass amount. According to the microbial community analysis, *Diphylleia rotans, Synechocystis* PCC-6803 and *Chlorella sorokiniana* were identified as mostly detected species after confirmation with 4 different markers. The abundance of *Chlorella sorokiniana* and *Synechocystis* PCC-6803 increased almost 2 folds both in iron and magnesium addition. On the other hand, the dominancy of Diphylleia rotans was not affected by iron addition while drastically decreased (95%) with magnesium addition. This study helps to understand how the dynamics of symbiotic life changes if macro elements are added to the ALD and reveal that microalgae can adapt to adverse environmental conditions by fostering the diversity with a positive effect on high value product.

In conclusion, in this thesis, the production of microalgae biomass have been successfully carried out, and the macronutrients needed in the production of microalgae have been provided from the digestate effluent obtained from a real-scale anaerobic digester using a mixture of domestic organic solid waste, industrial treatment sludge and animal feces. Moreover, it helped to understand how the dynamics of symbiotic life changes if macro elements are added to the ALD and reveal that microalgae can adapt to adverse environmental conditions by fostering the diversity with a positive effect on high value product. This thesis might help to be a

answer for aiming both reducing adverse effect of anaerobic liquid digestate by mixed microalgae and increasing product values by valorization and give an idea that digestate can be diluted with sea water instead of tap water in the future studies. Moreover, by this thesis, it was proves that digestate cultivated algae are rich source of primary (carbohydrates, proteins, lipids) and secondary (pigments) metabolites that could be exploited to produce biofuels, bio-polymers, biofertilizers, nutraceuticals, food/health grade compounds, enzymes, feed supplements etc. and the treated digestate can be reutilized for the agricultural or industrial purposes according to the high removal rates by mixed culture.

ANAEROBİK ÇÜRÜTÜCÜ ÇIKIŞ SUYUNDA BÜYÜTÜLEN MİKROALGLERDEN DEĞERLİ ÜRÜN GERİ KAZANIMI

ÖZET

Dünya nüfusunun ve bağlantılı olarak ihtiyaçlarının giderek artması sonucunda hızla gelişen endüstrileşme son yüzyılda ciddi bir enerji açığı yaratmıştır. Özellikle son yıllarda ihtiyaç duyulan bu enerjinin karşılanması için fosil yakıt kullanımının hızla arttığı ve bunun da yüksek miktarda sera gazı salınımına yol açtığı bilinen bir gerçektir. Bu konuda yapılan araştırmalar sera gazlarının küresel ısınmaya ve iklim değişikliğine yol açtığını ortaya koymaktadır. Ekolojik dengenin bozulmasıyla sonuçlanacağı bilinen etkileri görülmekte olan bu olumsuzluklar bilimsel araştırmaların yönünü alternatif çevreci enerji arayışına çevirmiştir.

Son yıllarda ağırlık verilen araştırma konularından biri de biyokütle ve çeşitli biyokütle kaynaklarından elde edilebilen biyoyakıtlardır. Biyokütle enerjisi, petrol ve kömür gibi güneş enerjisinin kimyasal bağlarda depolanmış halidir. Fotosentez yapan canlılar su, CO_2 ve makronutrientler kullanarak; inorganik maddeden organik maddeler sentezlerken güneş enerjisini biyokütlelerini meydana getiren bileşiklerdeki kimyasal bağ enerjisine dönüştürürler. Bu biyokütlelerin doğrudan ya da depolanmış bu enerjiden maksimum verim elde etmek amacıyla biyoyakıt olarak tanımlanan biyodizel, biyoetanol ve biyogaz gibi çeşitli bileşenlere dönüştürülerek kullanılması mümkündür. Bu biyokütlelerden elde edilen enerji; yenilenebilir enerji kaynakları olarak sınıflandırılmaktadır.

Biyokütle enerjisi karasal bitkilerden elde edilebileceği gibi sucul bitkilerden de elde edilebilmekle beraber son yıllarda yapılan araştırmalar mikroalg üretiminin karasal bitkilerle karşılaştırıldığında birçok yönden avantajlı olduğunu ortaya koymaktadır. Mikroalg biyokütlesinin biyoyakıt elde etmek amacıyla üretimi, temiz su gerektirmemesi, düşük karasal alan ihtiyacı, yakma gazı ve atıksu kullanımı imkânı ile dikkat çekmiştir.

Anaerobik çürütme, yenilenebilir kaynaklardan enerji üretmek için kullanılan en yaygın yöntemlerden biridir. Çürütme sonucu oluşan biyogaz, gelişmiş ülkelerde büyük ölçekte ısı ve güç üretmek için kullanılırken, gelişmekte olan ülkelerde mevcut hayvan gübresi yönetimindeki sorunları çözmek ve ısıtma amaçlı kullanılmaktadır. Bu yöntem, sera gazı emisyonlarının özellikle metan gazı salınımının azaltılmasına yardımcı olurken, toprak iyileştirme özelliği yüksek olan çürütücü çıkış atığının, tarımda kullanılabilmesi gibi faydaları da vardır. Fakat çürütücü çıkış atığının, bazı istenmeyen koku, viskozite, yüksek nem ve yüksek uçucu yağ asitleri gibi özellikleri, bitkilerde fitotoksik etki oluşturabilir. Bu nedenle arıtma yapılmadan tarımsal topraklarda uygulanması risk oluşturabilir. Ayrıca, eğer çürütücü hammaddesine ısıl işlem uygulanmazsa veya çürütücü termofilik şartlarda işletilmezse patojenler tarım arazisine uygulamada sıkıntı oluşturabilir.

Anaerobik çürüme sırasında, organik azotun amonyak azotuna ve toplam fosforun da ortofosfata dönüsmesi, çürütücü çıkış suyunda yüksek konsantrasyonlarda amonyak ve fosfor bulunmasina sebep olur. Çürütücü çıkış atığı için uygulanan en yaygın

yöntem, araziye doğrudan uygulanmasıdır. Çürütücü çıkış atığının doğrudan araziye uygulanması, tarımsal alanlar için gübre kullanımı (mineral ve organik madde) açısından ucuz bir yöntem olarak kabul edilmektedir. Özellikle organik madde içeriğinin yüksek olması bazı tarım arazilerinde istenen bir durumdur. Ancak, kolay ayrışabilir organik bileşikler açısından tam olarak tüketilmemiş bir çürütücü çıkış atığı da elde edilebilir. Bu durum depolama aşamasında sorunlar yaratabilir (koku emisyou, patojenlerin yeniden gelişmesi ve fitotoksisite) ve potansiyel gübre değerini kısıtlayarak toprak-bitki sistemi üzerinde olumsuz etkilere yol açabilir. Bunlar bitkiler üzerinde tohumların filizlenmesinde gecikmeler, bitki ölümleri veya büyümede gerilemeler gibi incelenmesi gereken büyük ölçeklerde zararlı etkilere yol açabilir. Tarımda çürütücü çıkışının atığı, tahıl üretiminde genelde kuzey avrupa ülkeleri, Danimarka, İsveç, İskoçya ve Almanya gibi ülkelerde kullanmaktadır. Ancak çürütücü çıkış atığının toprak düzenleyicisi veya gübre olarak kullanılması istenirse, Almanya ve İngiltere'deki çürütücü çıkış atığının uygulamasında kullanılan kalite protokolleri gibi Avrupa ve Ulusal kurallar tarafından düzenlenen kalite standartlarına uygunluğu denetlenmelidir.

Mikroalglerin özellikle protein olmak üzere yüksek besin içeriklerinden dolayı insanlar tarafından kullanılmaları eski Çin'de 2000 yıl kadar önceye dayanırken, mikroalg kültürü ve üretimleri 1950'lerde başlamıştır. O yıllarda mikroalg üretimi çoğunlukla besin ve beslenme yan ürünü elde etmeyi amaçlamış olsa da yanı sıra ilaç ve kozmetik sanayinde kullanılan değerli bir takım maddeler de alglerden elde edilen ürünler arasında yer almaktadır. Mikroalgler çoğunlukla fotootrofik olarak beslenen yani organik besinlerini güneş enerjisiyle inorganik maddelerden sentezleyen, tek hücreli mikroorganizmalar olarak tanımlanırlar. Mikroalglerin biyokütle kompozisyonları, depo maddeleri ve bu maddelerin oranlarının ortam koşullarına göre değiştiği bilinmektedir. Doğal şartlar altında fotosentez depo ürünleri karbonhidratlar olan mikroalg türlerinden birçoğunun istenmeyen stres koşullarına farklı adaptasyonlar geliştirdikleri ve depo materyallerinin kompozisyonlarının değiştiği gözlemlenmiştir. Özellikle başta N azot olmak üzere mineral eksikliği ve yüksek aydınlatma koşullarında sitoplazmik lipid granülleri halinde yağ biriktirdiği raporlanmaktadır. Hücrelerde biriktirilen yağın ayrıştırılıp biyodizele dönüşümünün mümkün olduğunun ortaya çıkmasıyla, mikroalglerden biyodizel üretimi çalışmaları hızlanmıştır.

Enerji elde etme amacıyla mikroalgal biyokütlenin kullanılmasının uygulanabilir hale gelmesi için üretim maliyetinin düşürülmesi konusunda birçok bilimsel araştırma sürdürülmektedir. Maliyeti arttıran önemli parametrelerden biri de mikroalglerin azot ve fosfor ihtiyacı olduğundan besi maddesi maliyetlerinin azaltılması için bu maddelerin atıksudan karşılanması konusu oldukça dikkat çekmektedir. Mikroalaglerin atıksu arıtımı amacıyla kullanımları ilk kez 1957 yılında denenmiş olmasına karşın, bugüne kadar evsel atıksularla az sayıda pilot ölçekli çalışma gerçekleşmiştir. Bu çalışmalar ile atıksulardan alg kullanılarak besin maddesi giderimi konusunda önemli bilgiler elde edilmesine karşın halihazırda uygulamaya yönelik bir çok soru ve bilinmezlik mevcuttur.

Çürütücü çıkış suyunda yetiştirilen mikroalglerden elde edilen pigmentler, proteinler, lipitler ve karbonhidratlar gibi yüksek değerli ürünler yakıt, gübre ve hayvan yemi olarak kullanılarak mikroalgal biyokütle üretiminin toplam maliyetinde önemli bir tasarruf sağlamaktadır. Bu kapsamda, gelişmiş arıtma yöntemleri ile arıtma yapmak yerine, anaerobik çürütücü çıkış suyunda başarılı büyümenin uygulanabilirliğini göstermek bu tezin başlıca hedefidir. Bu amaçla, anaerobik çürütücü çıkış suyunda

yetiştirilen karışık mikroalglerin arıtma verimi ve uygulanabilirliği araştırılmış, çürütücü çıkış suyunun algal kütlede bulunan yüksek değerli ürünler üzerindeki olumlu etkisi incelenmiştir. İTÜ Ayazağa Yerleşkesinde yer alan su kütlelerinden izole edilen yabani tür mikroalgler, gerçek arıtma tesisinden elde edilen anaerobik çürütücü çıkış suyunda yetiştirilerek mikroalg-bakteri bazlı arıtım sistemlerinin sürdürülebilirliğini teşvik etmek için yüksek değerli ürünler ayrıntılı olarak gözlemlenmiştir. Bu kaspamda yapılan çalışmalar bölümler halinde aşağıda özetlenmiştir.

Birinci bölümde, Michaelis-Menten denklemi kullanılarak Anaerobik Çürütücü Çıkış Suyunda (ACCS)'da büyüyen karışık mikroalglerin besin giderimi için biyokinetik katsayıları araştırması üzerine kesikli deneyler yapılmıştır. Başlangıç nütrient miktarları % 2, %5, %7 ve %10 seyreltme oranına karşılık gelecek sekilde NH_3-N konsantrasyonu 18.6–87.1 mg L^{-1} arasında değişirken, başlangıç PO_4-P konsantrasyonu 1.85–6.88 mg L^{-1} arasında belirlenmiştir. Verim sonuçlarına göre (mg chl a mg^{-1}), karışık mikroalglerin azotu fosfora kıyasla 10 kat faha fazla kullandiğı bulunmuştur. Biyokinetik katsayılar nitrojen için k_N = 2.48 mg NH_3-N mg^{-1} chl a d^{-1}, K_{mN} = 29.3 mg L^{-1}, Y_N = 0.45 mg chl a mg^{-1} NH_3-N; ve fosfor için k_P = 0.21 mg PO_4-P mg^{-1} chl a d^{-1}, K_{mP} = 2.94 mg L^{-1}, Y_P = 5.03 mg chl a mg^{-1} PO_4-P olarak belirlenmiştir. En yüksek klorofil miktari (39 mg L-1 veya 3.31 mg L^{-1} d^{-1}) %10'luk en yüksek seyreltme oranında gözlemlenmiştir. Buna ek olarak, en yüksek biyokütle (1.25 g L^{-1}) koyu renk, yüksek azot ve yüksek partikül miktarına rağmen en yüksek dilusyon oranı olaran 10%'da gözlemlenmiştir.

İkinci bölümde, *Chlorella vulgaris* ve *Scenedesmus armatus*'un hakim olduğu karışık mikroalgal kültür, 0 ila 100 mM arasında değişen farklı NaCl konsantrasyonları altında ACCS'de büyütülmüştür. Tuzluluk 50 mM NaCl olduğunda en yüksek lipid ve karbonhidrat miktarı sırasıyla % 38 ve % 36 olarak gözlenmiştir. Bununla birlikte, artan NaCl konsantrasyonu ile protein içeriği % 13'e düşmustur. Ayrıca alg biyokütlesi toplam klorofil miktarı ile birlikte artan NaCl konsantrasyonu ile birlikte azalmıştır. Alg türleri, tuzluluk stresine çeşitli tepkiler göstermiş ve ACCS'de karışık mikroalglerin yetiştirilmesine yönelik uygun maliyetli bir yaklaşım sergilemiştir ve gelecekteki çalışmalarda ACCS ve/veya diğer atık suların musluk suyu yerine deniz suyu ile seyreltilebileceği konusunda fikir vermiştir. Bu çalışma, yüksek lipid artışı nedeniyle, ACCS'yi değerlendirmeye ve ekonomiye kazandırmaya yardımcı olacak biyoyakıtlara dönüştürülebilir sonuçlar sunmuştur.

Üçüncü bölümde, ACCS'de yetiştirilen karışık mikroalglerin popülasyon dinamikleri, biyokimyasal kompozisyonu ve yağ asidi içeriği üzerindeki etkisini gözlemlemek için 0 ile 4.5 mM arasında değişen FeSO4 takviyesi ve 0 ile 5.1 mM arasında değişen $MgSO_4$ takviyesi incelenmiştir. Genel olarak, ACCS'ye 3.1 mM FeSO4 ilavesi, toplam protein içeriğini% 60 arttırmış ve en yüksek biyokütle (1.56 g L-1) ve klorofil-a miktarı (18.7 mg L^{-1}) gözlemlenmiştir. Buna ek olarak, 0,4 mM $MgSO_4$ ilavesi toplam karotenoid miktarını 2,2 kat artırırken, biyokütle miktarı üzerinde etkisi olmamıştır. Mikrobiyal topluluk analizine göre, *Diphylleia rotans, Synechocystis* PCC-6803 ve *Chlorella sorokiniana*, 4 farklı primer dizinleriyle onaylandıktan sonra en çok tespit edilen türler olduğundan emin olunmuştur. *Chlorella sorokiniana* ve *Synechocystis* PCC-6803'ün miktari hem demir hem de magnezyum ilavesinde neredeyse 2 kat artmıştır. Öte yandan, *Diphylleia rotans'ın* baskınlığı demir ilavesinden etkilenmezken, magnezyum ilavesiyle önemli ölçüde azalmıştır (% 95). Bu çalışma, ACCS'ye makro/mikro elementler eklenirse simbiyotik yaşamın dinamiklerinin nasıl değiştiğini anlamaya yardımcı olmuş ve mikroalglerin, çeşitliliğini artırarak yüksek

değerli ürün üzerinde olumlu bir etki yaratarak olumsuz çevresel koşullara uyum sağlayabileceğini ortaya çıkarmıştır.

Özetle, bu tezde biyokütle üretimine yönelik mikroalg biyokütlesi üretimi konusunda çalışmalar yürütülmüş, mikroalglerin üretilmesinde ihtiyaç duyulan makronütrientlerin, evsel organik katı atık, endüstriyel arıtma çamuru ve hayvan dışkısı karışımının kullanıldığı gerçek ölçekli bir anaerobik çürütücüden elde edilen çürütücü çıkış suyundan karşılanması sağlanmıştır. Sonuçlar gelecek vaat edici olup, karışık mikroalg ile hızlı besin giderme işlemlerinin uygulanabilirliği ortaya konmuştur. Böylece, çevre kirliliğinde ana kirleticiler olarak kabul edilen azot ve fosforun alg çoğaltılmasında kullanılması ile hem çevre kirliliği azaltılmış hem de alg büyümesi için dışarıdan temin edilen gerekli besin maddelerinin kullanımı ve maliyeti ortadan kaldırılmıştır. Buna ek olarak, sıvı fermente üründe yetistirilen alglere yapılan farklı stres koşullarıyla değerli ürün miktarı arttırımı, saf kültürden bağımsız karışık kültürün de değerli ürün geri kazanımında başarıyla kullanılabilecegine dair önemli sonuçlar vermiştir. Sonuç olarak, bu çalışma ile çoklu ürün geri kazanımına sahip alg biyokütlesine dayalı entegre sürdürülebilir arıtma başarıyla sağlanmıştır.

1. INTRODUCTION

1.1 Background

Although microalgae consumption by humans dates back in ancient China up to 2000 years ago - due to high nutrient content, especially for protein- culturing and production of microalgae only started in 1950s. In those years, microalgae production has mostly aimed to obtain nutritional and nutritional by-products, while valuable materials used in medicine and cosmetics industry are among the products obtained from algae (Spolaore et al., 2006).

Microalgae are often described as single-celled microorganisms, which are nutritionally photoautotrophic. It is known that microalgae's biomass compositions, storage materials and the proportions of these materials vary according to ambient conditions. Various scientific research are in progress to decrease the cost of production so that the use of microalgae biomass for bio-product production becomes feasible. One of the important parameters that increase the cost is that microalgae need nitrogen and phosphorus, so that it is very important to meet these substances from the wastewater in order to reduce the costs of the feedstock.

Algae based wastewater treatment processes have been gaining attention since 1960s because they could potentially offer many advantages (Wang et al., 2010), while reducing the cost of microalgae production. Many microalgae species such as green algae and cyanobacteria can absorb nitrogen and phosphate into their biomass as well as inorganic carbon for photosynthesis. A microalgae system can be used as an alternative secondary or post-secondary treatment process to remove nutrients from wastewater (Park and Craggs, 2010).

Bioremediation systems such as microalgae and bacterial wastewater treatment, and two-purpose systems such as extraction of biofuel and chemical products after waste water treatment, provide significant savings in the total cost of production of microalgae biomass. Furthermore, the integration of wastewater treatment with microalgae-based biofuels and bioproducts production has significant advantages to prevent environmental pollution. However, the highest yield microalgae production

varies according to the conditions such as the characteristic of wastewater, the original habitats of the algae or the growing environment conditions. Algae can multiply in many wastes such as domestic wastewater, animal wastes, agricultural-industrial wastes (Abdel-Raouf, 2012).

This digestion process successfully transforms biowastes into two economically useful by-products: a renewable energy source (biogas) and a potential fertilizer and soil amendment: the anaerobic digestate. The end-product digestate may involve issues such as odour emission, toxic organic compounds, pathogens and phytotoxicity. Also, this anaerobic digestate can have toxic effects on plants, eutrophication on water bodies and groundwater pollution due to the high concentrations of both ammonia and phosphorus present. Therefore, nutrient recovery is required to obtain high value from wastewater. In this scope, microalgae holds great potential in conducting the recovery of nitrogen and phosphorus from digestate. The cultivation of microalgae in digested effluents offers important benefits in terms of wastewater treatment, production of valuable biomass which can be further valorized, and also causes a substantial reduction in the upstream production expenses.

There is a wide array of anaerobic digestates of which their composition and nutrient content depend mainly on the type of feedstock and digestion process. Different types of feedstock and also combinations of various feedstocks have been reported, such as livestock manure, cattle, dairy manure and agricultural residues/biowastes, organic solid wastes and sewage sludges, food wastes and landscape wastes, along with potato and sisal pulp wastes.

Depending on the physicochemical characteristics of the produced biomass, a wide range of subsequent applications have been proposed due to the numerous benefits posssessed by microalgae in digested effluents. The buildup of high added-value products, including lipids, proteins, carbohydrates, and pigments, the formation of metabolites, and the formation of biofuels have been deeply stressed.

1.1.1 Microalgae and Wastewater

Accompanied by the reduction of energy resources and environmental weakening, water shortage is one of the world's most critical problems. The impact of current industrial development, worldwide mobility, and growing populace has greatly affected freshwater reserves globally. Wastewater sources can be classed as domestic,

agricultural and industrial effluents and the disposal of these effluents into water bodies risks harmful and dangerous impacts on human and animal health. This is due to the higher presence of nitrogen, phosphorus, sulphur, heavy metals and other organic/inorganic pollutants. Numerous traditional treatment techniques were used for treatment of wastewater, however, none of these methods can be proven as a widespread process due to their inefficiency, high expenses and non-environmently friendly nature. On the other hand, the use of microalgae for wastewater treatment offers a wide range of advantages in comparison to chemical-based treatment methods. Cultivation of microalgae employing wastewater results in the highest atmospheric carbon fixation rate (1.83 kg CO2/kg of biomass) alongside quickest biomass productivity (40-50% higher than terrestrial crops) amongst all terrestrial bio-remediators with associated contaminant removal (80-100%). Further to this, the algal biomass may also comprise of high value metabolites such as omega-3-fatty acids, pigments, amino acids, and high sugar content. Therefore, after extraction of these high-value beneficial components, residual biomass can be either directly converted to energy by means of thermochemical transformation or can be used for the production of biofuels by way of biological fermentation or transesterification.

Microalgae are commonly described as single-celled microorganisms, which are nutritionally photoautotrophic, ie, synthesize organic nutrients from inorganic and inorganic materials. Algae are one of the the most varied groups of eukaryotes. On average, there has been more than 350,000 microalgal species discovered globally (Shahid et al., 2017; Afzal et al., 2017). Furthermore, microalgae can be easily grown on minimal lands by using seawater or wastewater as growth media marginal, avoiding the need for large arable land areas (Miranda et al., 2017). According to the varying strains, microalgae can be utilised in numerous industries such as cosmetics, agriculture, biofertilizers, therapeutics along with green-fuels - namely bioalcohols, biogas and biodiesel (Afzal et al., 2017). However, it is necessary to develop cost-effective processes to obtain cost-competitive algal products, as in current times, algal biofuels are unable to compete with fossil fuel prices. Therefore, selection of suitable strain, use of inexpensive media, optimization of conditions resulting in higher biomass production, cell stoichiometry to divert the balance towards target product, suitable commercialization, and reducing the operational cost (mainly associated with cultivation and harvesting stages) are leading focused aspects of algal research.

Wastewater is the most suitable resource for high algal biomass production due to numerous reasons such as; (i) inexpensive growth media, (ii) encourages bulk biomass and biofuel production, (iii) can supply ample valued nutrients, and (iv) offers possibility of assimilating algal cultivation with existing infrastructure wastewater treatment. Several studies have been carried out in recent decades regarding microalgae-based phycoremediation of wastewater and biorefinery based methods have been proposed. Microalgae have been investigated for wastewater (both industrial and domestic) treatment including brewery wastewater, domestic wastewater, textile wastewater, pharmaceutical waste streams, slaughterhouse industry, heavy metal-containing wastewater, palm oil mill effluents, starch-containing textile wastewater, and agro-industrial wastewater.

Studies carried out with microalgae using differing wastewater sources show that nutrient removal and biomass production potentials vary from each other. However, it is assumed that work to be done on experimental testing of combinations of wastewaters and different algal species with environmental factors in varying characters, will result in the enrichment of literature and provide valuable contributions surrounding this topic. It is challenigng to sustain pure cultures under field conditions, and is even more challenging when employing pond sources which receive wastewater. Therefore, researchers have recently focused on the cultivation of mixed algal cultures. Binary cultures in the form of consortia (microalgae-microalgae or microalgae-bacteria) have been described extensively for enhanced wastewater treatment due to their much higher ability for nutrient removal along with enhanced biomass production. Polycultures can be combined with different metabolic practices to allow themselves to survive under environmental stress conditions which allow us to create vigourous biological systems for wastewater treatment. Further to this, integrated consortia can uptake nutrients at an increased rate (Johnson and Admassu, 2013) due to one strain responsible for removal of nitrogen and another strain responsible for the removal of heavy metals purposely. These consortia possess many advantages including (i) contamination and predator resistances due to production of allelochemicals (ii) enhanced nutrient consumption; guaranteeing adequate nutrient supply throughout whole process, (iii) development of settleable system for flocculation which eliminates limitations of harvesting, and (iv) enhanced viability of phycoremediation; as loss of one microbe is reimbursed by other species. However, it

is challenging to develop robust consortia as a wide array of combinations is possible, leading to some restrainsts surrouding this process. Alongside this, maintenance of consortia for longer periods especially in an open-pond system have proven to be difficult (Gonçalves et al., 2017). Microalgae have the ability to apply CO_2, produced by bacterial respiration, for the synthesization of organics such as sugars, acetate, and glycerol for heterotrophic growth of bacteria. Moreover, the cultivation of many photosynthetic microalgae requires vitamins such as B12, with vitamin B12 auxotrophs greatly prevalent amongst microalgae.

The symbiotic relationship of microalgae and bacteria may be in the form of commensalism, mutualism or parasitism. Generally, bacteria heterotrophically produce CO_2 and other important nutrients which are consumed by microalgae for their growth and development (Zhu et al., 2019). On the returning hand, oxygen produced by microalgae during photosynthesis is valuable and utilised by bacteria (Rashid et al., 2018). Moreover, bacteria supply growth-promoting hormones and vitamin B to microalgae which are necessary for development (Fuentes et al., 2016). Additionally, this symbiotic relationship offers protection to the microalgae from other invasive species. However, bacteria may damage and disrupt the microalgal cell wall to use intracellular nutrients (Magdouli et al., 2016). This specific property is of great interest during harvesting stages where cell rupturing is necessary to obtain the target product and therefore, reducing expenses and timing of downstream processing in the biorefinery. The MB consortia also utilise dead algal cells as a nutrient source (Ramanan et al., 2016). Release of specific chemicals by bacteria and microalgae may also suppress the growth of each other (López-Serna et al., 2019). This property can be beneficial in regards to the reduction of sterilization cost as it eradicates the contamination possibilities (Kouzuma and Watanabe, 2015).

To conclude, it is known that microalgae's biomass compositions, storage materials and the proportions of these materials vary widely according to ambient conditions. Several scientific studies are being undertaken in an attempt to reduce the cost of production leading to the use of microalgae biomass for bio-product production becoming achievable. One of the vital parameters which increases the expenses, is that microalgae require nitrogen and phosphorus, and therefore, it is very important to obtain these substances from the wastewater source in order to reduce the costs of the feedstock.

1.1.2 Microalgae and Digestate

The anaerobic digestion process was originally highlighted for the first time by Alessandro Volta in 1776. Since then, this process has been majorly applied mainly for biogas production and for waste treatment. Because of increasing energy prices along with negative impacts of fossil fuels on global warming and population surge, popularity of the biogas process has grown immensely since the 1970s. Anaerobic digestion is defined as a controllable, biological process, in which a variety of anaerobic microorganisms utilise organic matter as substrate to produce methane (CH_4) and/or hydrogen (H_2) in the absence of oxygen (Jain et al., 2015). Anaerobic digestion consists of four consecutive biological processes (Table 1.1): hydrolysis, acidogenesis, acetogenesis and methanogenesis (McKennedy and Sherlock, 2015). Firstly, hydrolysis involves complex organic matters such as carbohydrates, proteins and lipids being broken down into soluble derivatives – aided by extracellular enzymes which are excreted by various bacteria. In the following two processes, acidogenesis and acetogenesis, the already hydrolyzed molecules, which include sugars and amino acids, are converted into CO_2, H_2, NH_4–N and organic acids by acidogenic bacteria. Next, the subsequent organic acids are continuously converted into acetic acid, along with additional NH_4–N, H_2 and CO_2. During the final phase, methanogenic archaea use the intermediate products of the previous phases and convert them into CO_2, water and CH_4. During the digestion process, approximately 20–95% of the feedstock organic matter is degraded. Nitrogen is converted to NH_4, however, the majority of both N and P are conserved. This allows the N & P content of the resultant digestate to be typically comparable to that of the feedstock material (Provenzano et al. 2011).

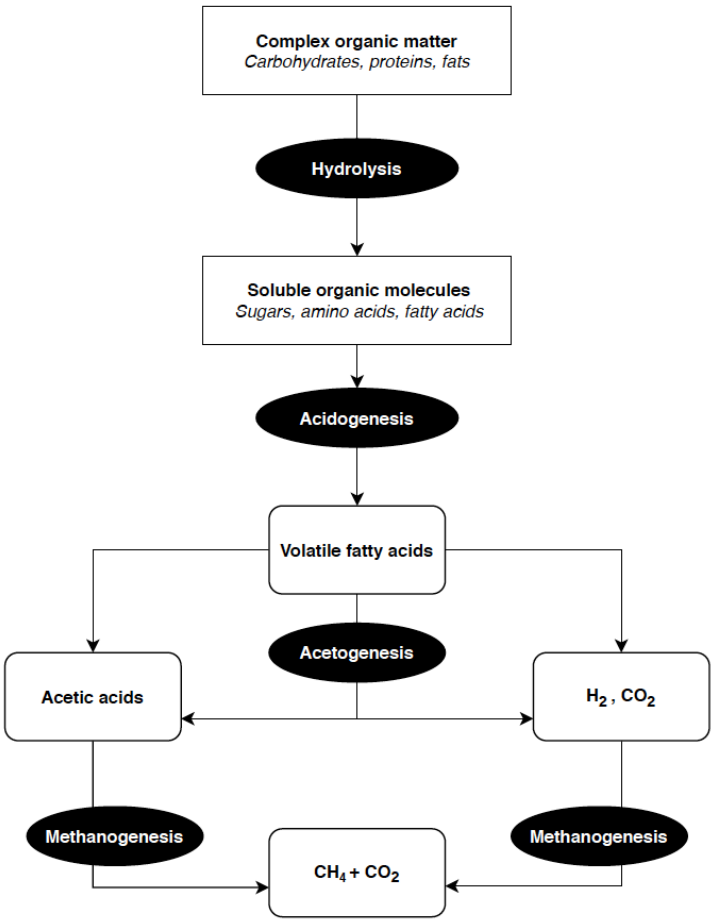

Figure 1.1 : Anaerobic digestion process.

This process successfully convert biowastes into two economically useful by-products: a renewable energy source (biogas) and a potential fertilizer and soil amendment: the anaerobic digestate. The end-product digestate may have issues such as odour emission, toxic organic compounds, pathogens and phytotoxicity. Moreover, it can have toxic effects on plants, eutrophication on water bodies and groundwater pollution due to the high concentrations of ammonia and phosphorus present in the anaerobic digestate. Therefore, nutrient recovery is necessary to have high value from wastewater. In this scope, microalgae is a great candidate for the recovery of nitrogen and phosphorus from digestate. Cultivating microalgae in digested effluents offers significant advantages in terms of wastewater treatment, the production of valuable biomass which can be further valorized, and a significant reduction in the upstream cost of the process.

There is a wide range of anaerobic digestates whose composition and nutrient content depend upon the primarily on the nature of the feedstock and the digestion process.

Various types of feedstock and combinations of feedstocks have been reported, such as cattle manure, livestock manure and agricultural residues, organic solid wastes and sewage sludges, dairy manure and biowastes, food wastes and landscape wastes, and potato and sisal pulp wastes.

The majority of studies demonstrate that digestates are richer, in terms of nutrient contents, than their respective raw manure counterparts. The organic N portion in the excrements is further digested in the bioreactor where retention times are much longer than in comparison to animal digestive tracts. This may explain the higher amount of NH_4-N generally observed in digested as compared to undigested slurries. When anaerobic digestates are added into soils, NH_4^+ is either absorbed by plant root cells, or adsorbed onto negatively charged soil particles, or oxidized to NO_3^- by nitrifying microorganisms. Nutrient leaching potential following application of anaerobic digestates is dependable upon various factors such as fertilisation strategies (e.g. time and methods of application), soil texture (e.g. sandy and clayey soils), topography, precipitations and cropping systems. Best management practices that can be employed to alleviate nutrient leaching include: alteration of digestate nutrient supply to crop demand and soil tests, synchronization of nutrient release with crop developmental demand, cultivation against slopes, avoidance of fall applications, lengthly period gaps between digestate employment and sowing, and applications that undertaken before heavy rains.

The microbiological status of the output digestate is dependant on the quality of the input feedstock and on the configuration characteristics of the digester such as pre-treatment (pasteurisation), digestion temperature, pH, ammonia concentration, hydraulic retention time, for example. The tenacity of various pathogens in digestates can be explained by the presence of bacteria species possessing the ability of forming spores amongst animal wastes. These spore-formers are not eradicated during the anaerobic digestion process. Regrowth of pathogens and their spores is possible in storage facilities. Stabilization of digestates through post-treatment measures such as curing (Drennan and DiStefano 2010) and composting (Tiquia et al. 1996) substantially decreases the risk they pose on human health and the environment. Typical nitrogen and phosphorus concentrations range between 5 and 15 kgN/ton and 0.1 and 1 kgP/ton respectively in cattle and chicken manure (Giuliano et al., 2013).

These nutrients remain in digestate after anaerobic digestion and, follwing an adequate treatment, can be recovered in a concentrated form of which can be easily transported.

Digestates can be abundant in a number of macro nutrients (e.g. N, P, K, S, Mg, Ca, Fe, and Na) and may also comprise of a number of trace elements (e.g. Co, Fe, Se, Mo and Ni) either as a result of the initial feedstock used (Marcato et al. 2008), or becaue of supplementation, addition of trace elements for enhanced digester performance (Williams et al. 2013). Digestate can be divided into solid and liquid fractions. The liquid digestate contains less than 15% DM content, whereas the solid digestate contains more than 15% DM. Solid digestate can be employed similar to the composts or could potentially be composted with other organic residues, which can be more economically conveyed over larger distances than liquid material (Møller et al., 2000). This separation allows for the treatment or valorization of each fraction by mechanical, physicochemical or biological means. Liquid digestate generally has a high nutrient status, midway in strength between livestock manures and inorganic fertiliser (Nkoa, 2014). Digestate comprises of significantly more available N than cattle slurry (80 – 90 % of N in whole or liquor digestate). Although the compound form of N in digestate is more readily accesible for uptake by plants, environmental losses may occur following land application, which poses particular risks in areas where N is in abundance. Digestate, which is plentiful in valuable nutrients, can therefore be directly employed as a renewable fertilizer due to its contents of stable organic carbon and nutrients. Otherwise, when an excess of nutrients occurs in a given region, the digestate can be further treated to recover nutrients in concentrated forms, allowing them to be translocated at sustainable prices in various agricultural areas. However, a key environmental concern with land application of digestates is the potential contamination of surface and ground waters, alongside the eutrophication of water bodies with abundant volumes of nitrogen and phosphorus. Many different commercial options for digestate treatment and nutrients recovery are available, with the most relevant involving drying, stripping, evaporation and membranes technology. These processes have been commonly applied in recent times with alternate success for the treatment of anaerobic digestate or its solid/liquid fraction.

The composition of digestates differs extensively with regards to the feedstock, inoculum source, and operating conditions of AD (e.g., temperature, pH, hydraulic retention time). The great amounts of potentially toxic effluents, due to the presence

of non-biodegradable compounds such as recalcitrant organic molecules, high ammonia concentration, and heavy metals that become available, together with the rising storage and transportation expenses, lead to the requirement of implementation of appropriate management practices. Along with conventional agricultural uses of digestates, an integrated method including AD effluents and microalgal technology can effectively be employed. Cultivating microalgae in digested effluents presents significant benefits in regards to wastewater treatment, the production of valuable biomass which can be further valorized, and a significant reduction in upstreaming expenses. According to physicochemical characteristics of the produced biomass, a wide array of subsequent applications have been proposed. The collection of high added-value products, including lipids, proteins, carbohydrates, and pigments, the formation of metabolites, and the formation of biofuels have been highlighted. Nonetheless, numerous limitations regarding the quantitative and qualitative parameters of the final product, as well as legislative issues, should be considered in the design of a profitable and sustainable process.

Generally, the initial step of each digestate treatment procedure involves the physical solid–liquid separation. Normally, 40 to 86% of the organic matter is present in the solid fraction, while the liquid phase is comprised of a low organic matter content. The solid fraction contains approximately 75% of phosphorus, which is directly absorbed or trapped with calcium, magnesium, and nitrogen. The digestate liquid is categorized by low organic matter and phosphorus concentrations, counterbalanced by increased potassium and nitrogen concentrations (up to 80% in the form of ammonium). Typically, the resulting solid manure high in dry matter (DM) and the liquid manure low in DM obtained is directly employed as fertilizer (Table 1.1).

Table 1.1 : Parameters of digestates and liquid fraction.

Parameter	Digestates	Liquid fraction
pH	7.3-9.0	7.9
Total solids (%)	1.5-13.2	4.5-6.6
COD	210-6900	
Total inorganic carbon (mg L^{-1})	940-1350	
Volatile solids (% DM)	63.8-75.0	-
Total N (g Kg^{-1} FM)	1.20-9.10	4.0-5.1
Total NH_4 (g Kg^{-1} FM)	1.5-6.8	1.8-3.0
NH_4^+ share on total N (%)	44-81	40-80
Total C content (% DM)	36.0-45.0	48
C/N ratio	3.0-8.5	3.7-4.8
Total P (g Kg^{-1} FM)	0.4-2.6	0.7-1.0
Water-soluble P (% of total P)	25-45	-
Total K (% DM)	1.9-4.3	3.5-5.2
Total K (g Kg^{-1} FM)	1.2-11.5	-
Total Mg (g Kg^{-1} FM)	0.3-0.7	7.9
Total Ca (g Kg^{-1} FM)	1.0-2.3	-
Total S (g Kg^{-1} FM)	0.2-0.4	-

Studies examining the incorporation of algal growth and AD were conducted as far back as the 1950s (Golueke et al. 1957), with the topic once again gaining great momentum in the bioenergy sector. In recent times, the increasing demand for food, energy, and valuable chemicals has required the need for research and development on renewable, novel, and sustainable sources. Recycling nutrients from digestate with algal technology is still at an early intial phase. Combination of microalgae growth with liquid digestate treatment is both an ecologically and economically friendly technology for cheap biomass production utilised in further valuable applications. In microalgal technology, among the various sources of wastewater which have the ability of displacing synthetic nutrients, digestates have the potential to play a vital role; where several strategies should be applied to overcome the challenges in microalgal growth associated with digestate turbidity, ammonia toxicity, or

phosphorus limitation. In this aspect, this chapter will discuss obstacles and opportunities associated with developent of this new technology and provide alternative strategies towards the cultivation of mixed microalgae consortia, which could be further converted into biofuels and value added chemicals with a biorefinery understanding along with the digestate treatment.

Amongst the many thousands of microalgal species exisiting in nature, only a few commonly occurring species are currently studied and proven to be robust survivors in digestate. Both freshwater microalgae (*Scenedesmus* sp. and *Chlorella* sp.) and marine microalgae (*Nannochloris* sp.) have successfully eliminated nutrients from liquid digestate. Similarilly, Dunaliella sp., a specific microalga adapted to extreme environmental conditions, has also proven effective for nutrient removal in highly concentrated liquid digestates. Microalga species belonging to the genera Chlorella, Scenedesmus, and Desmodesmus, alongside key species, Chlorella vulgaris and Scenedesmus obliquus, allow the essential features of digestate and can meet the nutrient requirements of microalgae during the cultivation, and thus the digestate can serve as a nutrient-rich, organic fertilizer for the production of microalgae. Nitrogen sources capable of being absorbed by microalgal cells include: NH_4–N, nitrite, nitrate and simple organic nitrogen like amino acids; microalgal cells favor NH_4–N and can incorporate it much quicker (Kumar et al., 2010). NH_4–N in both ionized and unionized forms has the ability to be absorbed directly, and pH value of the culture is the core factor that affects transformation. However, the uptake of phosphorus is not intricate, and phosphorus can be proficiently absorbed by microalgal cells in the form of phosphate.

It is predominantly ammonia levels that cause inhibition in the anaerobic digestion effluent – which are usually quite high as microalgae can not survive. Due to this, a dilution is often required before the microalgae are nourished by the effluent. Biomass productivity, lipid content, and nutrient removal efficiency have been stated to differ on the dilution ratio (Cai et al., 2013). Golueke and Oswald (1959) succeeded in cultivating the *Scenedesmus* strain in digesate. Olguín et al.(1994) examined the growth rate of *Spirulina maxima* in the seawater-diluted swine anaerobic digestate effluent. These studies determined that CO_2 reinforcement increased cell growth yields and the combination of anaerobic digestion and algae cultivation systems can be deemed a viable method for nutrient recycling and wastewater improvement. Blier et

al. (1995) reported that *Phormidium bohneri* (cyanobacteria) and *Micractinium pusillum* (microalgae) species had the potential to grow in the anaerobic digestion effluent from a cheese factory and that 100% inorganic nutrient removal was delivered by this study. Bjornsson et al. (2013) conveyed the growth rates of the *Scenedesmus* strain in cattle and pig digestate effluent which was diluted with lake water and distilled water at varying ratios. The results determined that the highest growth rate occurred in the digestive effluent diluted with lake water. Succeeding experiments have also shown that the rate of growth and biomass yields of algae grown at the swine digestate effluent are higher in Mg^{2+} supplemented systems. In this experiment, it is understood that Mg^{2+} enters the system with the lake water, indicating that the Mg^{2+} element may be a vital nutrient for large biomass production. In support of these results, Park et al. (2010) observed that cell growth of cultures of *Scenedesmus acuminatus*, which is semi-continuously grown in pig wastes, is amplified by Mg^{2+} addition. Franchino et al. (2013) studied the growth of 3 different algal species (*Neochloris oleoabundans, Chlorella vulgaris* and *Scenedesmus obliquus*) in the anaerobic digestate effluent of cattle sludge and raw whey. The wastewater was diluted at various ratios and utilised as growth medium for microalgae. The results determined that all strains reached the maximum growth rate at 1:10 dilution. In another study conducted, Prajapati et al. (2014) employed anaerobic digestate effluent at certain diluted rates to cultivate *Chroococcus*. The results revealed that the digestate effluent diluted with 30% BG-11 medium proved as the optimal growth medium for the *Chroococcus* species (0.79 ± 0.064 g L^{-1}). Studies have determined that sludge digestate decantation is suitable for algae cultivation in liquid phase and other wastewater streams. Other studies related to algae cultivation in the digestate include:

Kim et al. (2015) determines that microalgae cultivation and microalgae technology can be carried out together for both wastewater treatment and biodiesel production. The *Scenedesmus* strain was observed to be an appropriate microalgae species to be cultivated in anaerobic digester (AD) effluent with low carbon concentration and high nutrient content (NH_3-N = 273 mg L^{-1}, total phosphorus (TP) = 58.75 mg L^{-1}). In studies carried out utilising this specific algal species, the removal efficiencies of the nutrients were resulted as 99.19% and 98.01% for nitrogen and phosphorus, respectively.

Massa et al. (2017) performed studies on the growth of two freshwater microalgae species *Tetradesmus obliquus* and *Botryococcus braunii*, the marine diatoms *Phaeodactylum tricornutum* and the photosynthetic cyanobacteria species *Arthrospira maxima* in the anaerobic digestion effluent. Anaerobic digestion effluent was acquired as a result of anaerobically decomposed zootechnical, vegetable and household wastes obtained from three differing sources. *A. maxima* and *T. obliquus* species for the ammonium nitrogen removal were determined to be more successful in comparison to other species, with a removal performance of 98.9-99.8%. Ammonium nitrogen removal for *P. tricornutum* and *B. braunii* species were resulted as 79% and 88.5%, respectively. Additionally, an increase in carbohydrate, lipid and unviable microalgae wastes due to co-growth of the *A. maxima* and *T. obliquus* species, which are continuously inoculated in the batch photobioreactor, has been noticed. Once the biomass composition is considered, probable applications are projected for use in the feed, energy and chemistry industries.

Marta Franchino (2016) conducted studies regarding the growth of the green algal species *Chlorella vulgaris* strain in diluted anaerobic digestion effluent. The anaerobic digestion effluent utilised throughout this study is anaerobic digestion of faeces and corn residues obtained from swine farms. Because toxicity and high amounts of nitrogen and phosphorus are present in these wastes, this study was aimed to reduce toxicity by utilising microalgae and successfully conducting recovery of nutrients. From this study, the possiblity to grow microalgae with high dilution of the effluent was concluded, and the results determined that in excess of 90% of ammonia, total nitrogen and phosphate removal were observed. However, it has been noticed that following a few days of running the system, growth due to phosphorus deficiency was limited.

Bjornsson et al. (2013) examine the growth rate of *Scenedesmus* sp. in anaerobic effluent containing bovine and swine fertilizers diluted with pure water and lake water to a varying extent. The results prove that the highest growth rate is within the effluent stream diluted with lake water. Further to this, studies have determined that growth rate and microalgae biomass amount are more plentiful when supplemented with Mg^{2+}. In this study, it was proven that Mg^{2+} enters the system with lake water and that perhaps Mg^{2+} may be the crucial nutrient element for high biomass productivity. This

determination was supported by Park et al. (2010), where an observation of the growth of Scenedesmus acuminatus in pig tail wastes was improved by Mg^{2+} addition.

Franchino et al. (2013) observed three different species of microalgae (*Neochloris oleoabundans, Chlorella vulgaris* and *Scenedesmus obliques*) in raw whey crude anaerobic digestion effluent and in bovine waste. Wastewater diluted in specific proportions is utilised as a growth medium for microalgae. According to the results, all species reached maximum growth rate when the dilution is 1:10.

According to another study, Prajapati and colleagues (2014) employed digestion effluent at varying rates to grow *Chroococcus*. The results determined that the digestion effluent was the most suitable medium for growth of *Chroococcus* when diluted with 30% BG-11 media (0.79 ± 0.064 g L^{-1}).

The results of Uggetti et al. (2013) proposed that microalgae biomass production of digestion effluent may have potential to be an adequate substrate for up to 2.6 g TSS L^{-1}. Biomass production is positively enhanced by the substrate concentration, while the microalgal growth rate is negatively influenced by self-shadowing. Hence, the micro algal initial concentration and the initial growth rate of the digestion effluent (μ: 0.9 to 0.04 d-1) are decreased, but biomass production is substantially increased (0.1 to 2.6 gTSS L^{-1}). Sharma (2010) determined that the consumption of nitrogen is founded on the Monod kinetics of modeling the effect. The net specific growth rate for *Chlorella pyrenoidosa* resulted as 0.6 day^{-1} while the doubling time was 1.15 days. Microalgal growth kinetics were discovered by reasonably observing the Monod growth model.

Invetigations conducted by Aslan and Kapdan (2006) regarding the effect of initial nitrogen and phosphorus concentration on the nutrient removal performance of *Chlorella vulgaris* strain and determination of the biokinetic constants using the Michaelis-Menten expression, such as reaction rate constant (k), half saturation constant (Km), and yield constant (Y), involved batch experiments were carried out. Experimental results obtained from this study determined that effluent quality is noticeably reduced with increasing nutrient concentrations and that algal cultures more efficiently remove nitrogen in comparison to phosphorus. Biokinetic coefficients for nitrogen are as follows: k = 1.5 mg NH4-N mg-1 chl a d^{-1}, Km = 31.5 mg^{-1}, YN = 0.15

mg chl a mg^{-1} NH_4-N; 1, Km = 10.5 mg L^{-1}, YP = 0.14 mg chl a mg^{-1} PO_4-P for phosphorus.

Bohutskyi et al. (2016) stated that wastewater served with %5-10 anaerobic digestion effluent (AD) shows higher optical density (OD) measurements. It was also concluded that microalgae growth rates increased from 0.2-0.3 $days^{-1}$ to 0.7-0.9 $days^{-1}$ for secondary effluent and from 10-20 mg L^{-1} to about 40-60 mg L^{-1} for biomass efficiency. It has been observed that there is a significant increase in nutrient concentrations(%100 for N; P, Mn; B; Zn, Co and %20-60 for S, Mg, Ca, Mo) in wastewater supported by anaerobic digestion effluent (AD) and that N: P ratio is improved positively. However, feeding of anaerobic effluent at 20% diution ratio, microalgae growth was affected negatively due to ammonia toxicity.

Malec et al. (2016) observed an effective proliferation of both *Chlorella* and other microalgae species in anaerobic effluent dilution (25-50%). The results show that the high initial concentration of ammonia and the low concentration of phosphorus may limit microalgae growth.

Koutra et al. (2016) examined the growth of two microalgae species, *Parachlorella kessleri* and *Acutodesmus obliquus* species, in their anaerobic effluent during their work. The anaerobic effluent used in the study was obtained anaerobically as a result of digestion of agricultural-industrial wastes and finished dairy products. The performance of the system was monitored by biomass, lipid production and nutrient removal in different anaerobic effluent loads, in situ and non-situ conditions. Growth was not observed after 9-12 days because the volume was caused by inhibition of growth of 10% anaerobic effluent water load. However, for biomass yields *P. kessleri* and *A. obliquus* were 1.1 g L^{-1} and 1 g L^{-1}; fatty acid concentrations were 22.7% and 19.5%, as well as ammonia nitrogen consumption were 49.7 mg L^{-1} and 32.3 mg L^{-1} and total phosphorus removal were 84.2% and 84%, respectively. In addition, studies have shown that there is no difference between the sterile and nonsterile groups.

Ledda et al. (2016) aimed at reducing the amount of wastewater resulting in an anaerobic digestion of the feces from the milk production facility by microalgae production and wastewater treatment; thereby affecting the energy balance of the system in the positive direction. *Scenedesmus* species were used in the studies and anaerobic digestion effluent as growth medium was subjected to ultrafiltration process

without subjecting to any purification treatment. It has been observed that the growth of the Scenedesmus strain used in the anaerobic digestion effluent exceeds 10%, causing inhibition. But below this value, productivity of only 124 mg L^{-1} could be achieved. Furthermore, the structure of the culture medium directly affects the biomass composition depending on the concentration of ammonia in terms of protein, carbohydrate and lipid content. The integration of microalgae production into the anaerobic digestion effluent is possible with a yield of microalgae biomass of 166 to 190 tons per year. When the general energy demand and mass balance of the system are examined, it is concluded that anaerobic effluent and microalgae production are applicable.

Hajar et al. (2016) conducted studies on the dilution of diluted anaerobic digestion effluent in order to use *Neochloris oleoabundans* strain as a potential biofuel raw material in a sustainable way. Laboratory-scale systems are operated with anaerobic digestion effluent at different dilution ratios and filtration. The highest growth was observed for the *N. oleoabundans* species with a dilution of 2.29% with 100 mgN L^{-1}.

The studies mentioned above show that the liquid phase media obtained from the sludge digestion plant and other waste streamsare suitable for microalgae growth.

Massa et al. (2017) observed the growth of *Tetradesmus obliquus, Botryococcus braunii, Phaeodactylum tricornutum* and *Arthrospira maxima* species in anaerobic digestate effluent. The digestion effluent was obtained by anaerobic digestion of 3 different organic wastes;zootechnical digestion effluent, anaerobic digestate effluent of plant biomass, and digestion effluent of organic fraction in domestic solid wastes. While zootechnical digestion effluent only affected *T. obliquus* and *B. braunii* growth, all strains showed the same growth performance in vegetative biomass digestion effluent as in the corresponding standard media. The digestion effluent from the organic fraction in domestic solid wastes was the lowest growth medium of all strains. When the nutrient removal efficiencies were examined, *A. maxima* and *T. obliquus* removed NH^{4+} -N between 98.9-99.8%, whereas the yields of *P. tricornutum* and *B. braunii* were 79.0% and 88.5%, respectively. Repeated fed-batch production in photobioreactors has shown an increase in the amount of lipid, carbohydrate and ash in the biochemical composition of *A. maxima* and *T. obliquus* biomass grown in the zootechnical digestion effluent and plant biomass digestion effluent.

Swine derived decaying effluent is toxic to many microorganisms due to the high amount of ammonia. Various studies have shown that mixed cultures containing at least three microalgae species can grow on undiluted swine-derived digestion effluent. Using the algae ponds, the mixed cultures were grown in the outdoor environment and the potential removal of NH_4-N was 63.7 ± 12.1 mg $N\text{-}NH_4^+$ L^{-1} d^{-1} was observed. In the study, mixed culture was dominated by *Chlorella* sp. and has a constant growth between 800 and 1600 mg $N\text{-}NH_4^+$ L^{-1}. In addition, microalgae have been observed to increase chlorophyll content by CO_2 addition to the pools to raise the pH to 8 (Ayre, J. M, et al., 2017).

In another study, it was observed that the production of *Nannochloropsis* strain could be successfully achieved in the digestion effluent from food waste. It has been said that biochemical compositions, including growth and fatty acid content, can be compared to the results of cultures produced using defined laboratory environments. The N:P ratio of the environment, including the digestion effluent, could be increased to 32:1 without significant effect, but more could reduce phosphorus use (Mayers, J. vd., 2017).

Nwoba et al. (2017) investigated the growth potential, nutrient removal rates and biochemical composition of biomass in open-air climatic conditions in different freshwater macroalgae in swine-derived digestion effluent. *Rhizoclonium* sp. and *Ulothrix* sp. were isolated from the mixed culture and grown efficiently in the digestive effluent from swine waste. Total carbohydrate and protein contents ranged between 42.8-54.8% and 43.4-45.0%, respectively, while total lipid content was observed to be very low. This study has shown that macroalgae cultures can be used as a source of animal feed production, as well as for the treatment of swine digestion effluent.

The anaerobic digestion effluent from kitchen waste is a potential waste water for microalgae growing because it contains plenty of nutrients. In this study, *Chlorella sorokiniana* SDEC-18 and *Scenedesmus SDEC-8* species were grown in anaerobic digestate effluent from kitchen waste. Results showed a production of 0.42 g L^{-1} and 0.55 g L^{-1} biomass in the anaerobic digestion effluent from kitchen waste, diluted 1/15. The lipid content of *Chlorella sorokiniana* SDEC-18 (30.27-41.69%) and *Scenedesmus SDEC-8* (35.97-47.39%) were found to have significant advantages over controls grown in BG11 medium in this medium (Zhang et al., 2017).

In Silkina et al. (2017)'ss work, the anaerobic digestion effluent of agricultural wastes were purified by membrane filtration to make N: P ratios 16.53, 3.78 and 14.22, respectively. Three algal species were cultivated with efficient bioremediation and high biomass. *Nannochloropsis oceanica* and *Scenedesmus quadricuada* have been reported to be an ideal production environment for lipid and biomass formation *for Schizochytrium limacinum* SR21 (16.70 w / w% and 1.42 g L^{-1}), while ammonia and phosphate removal were over 60%.

In a study by Hajar et.al. (2017), *Scenedesmus dimorphus* was grown in non-sterilized digestion effluent as a nutrient medium. *S. dimorphus* was grown in laboratory scale dilution rates of 0.05-10%. Dilutions of 1.25-2.5%, equivalent to a total nitrogen concentration of 50-100 mg N L^{-1} and a total phosphorus concentration of 6-12 mg P L^{-1}, provide sufficient nutrients to maximize the growth rate and also provide algal biomass at high concentrations. In the following studies, microalgae culture was scaled up to 100 L algae ponds and the effect of the pedal full mixing effect on growth was investigated. The maximum mass (0.446 g L^{-1}) was reached with a dilution of 2.5%; and 65-72%, 63-100% and 78-82% nitrogen, phosphorus and COD removal was observed, respectively.

In the study of Szwaja et al. (2017), nutrient removal by *Chlorella* sp was evaluated in anaerobic digested effluent.The results showed that *Chlorella sp*. biomass reached 386.5 ± 24.1 mg dry weight L^{-1} when grown in municipal anaerobic digestate wastewater. Lower ($p < 0.05$) microalgae growths were obtained in corn silage, molasses slurry and cattle sludge digestion effluent waters. Increasing the initial ammonia nitrogen concentration in the digester effluent to 160 mg L^{-1} has not increase the growth of *Chlorella* sp. due to phosphorus restriction. Ammonia nitrogen, total nitrogen, total phosphorus and chemical oxygen demand (COD) removal efficiencies reached 99.7%, 98.6%, 88.2% and 58.7%, respectively, depending on the digestion effluent source.

Yang et al. (2017) studied the ability of four oil microalgae isolated from freshwater to remove nitrogen and phosphorus. As food source, two types of anaerobic digested wastewater were used: grass anaerobically digested effluent and molasses wastewater anaerobically digested effluent. These wastewaters were diluted with BG-11 medium or tap water. The optimal growth medium for the GN 171 strain was determined as grass effluent diluted with tap water at a rate of 3.2 g L^{-1}, which was not sterilized.

Total lipid, carbohydrate and protein contents were found to be 34%, 30% and 16%, respectively. Total nitrogen, ammonia nitrogen, phosphorus and materials for removal of selected heavy metals were stated to be sufficient. As a result, it is possible to utilize extreme anaerobic digestion effluent from artificial medium for microalgae production.

Dilution of digestates with water is one of the most conventional methods in order not only to avoid the toxic effect of ammonia, but also to enable light penetration into the microalgae (Cai et al., 2013). Dilution ratios of digested effluents vary on numerous reasons, including the species which are used, the physico-chemical characteristics of digestates, along with the desired outcome of the microalgae (Singh et al., 2011; Khanh et al., 2013). For example, lipids amount can significantly improve during microalgal growth under nutrient-depleted conditions (Rodolfi et al., 2009). However, high dilution of digestate should be prevented (Franchino et al., 2016), since large volumes of water are required to dilute small volumes of effluents, making this procedure unsustainable. In a large scale system, 10% digestate concentration would be considered as relatively adequate for effective management of digested wastewaters (Franchino et al., 2013).

The residual effluent from the digestion process contains high amounts of nutrients, such as ammonium and phosphate. This effluent may generate alarms of pollution such as eutrophication when applied to environment, or financial problems if additional treatment is required preceding to discharge. However, the nutrient-rich effluent can be utilized as a growth medium for microalgae. Therefore, existing processes for AD may be coupled with microalgae cultivation to relieve costs associated with effluent treatment. The level of inhibitors – mainly ammonia – in the AD effluent is usually too high to be tolerated by microalgae. As a result, dilution is generally needed before the effluent is fed to the microalgae. Wang et al. (2010) studied the effectiveness of using digested dairy manure as a nutrient supplement for the cultivation of microalgae *Chlorella* sp. It was reported that biomass productivity, lipid content, and nutrient removal efficiency were all dependent on the dilution ratio (Cai et al., 2013). Other studies related to algae cultivation in the digestate effluent are given in Table 1.2.

Table 1.2 : Microalgae species grown in anaerobic digestate.

Digester feed composition	Characterization of raw digestate (g L^{-1})	Dilution ratio (%)	Microalgae Species	Valuable Products	Working pH	μ (day^{-1})	Highest microalgae biomass (g L^{-1})	References
Poultry litter	*Unfiltered* *NH_4-N: 0.87, BOD:3.98, COD:0.29,NO_3-N:0.02,*	25	*Auxenochlorella protothecoides*	Biomass	7.2	-	0.292*	Bankston and Higgins (2020)
Maize silage	*Unfiltered* *TN:0.15, NH_4-N: 0.85, BOD:3.98, COD:9.14,NO_3-N:0.04,*	20	*Auxenochlorella protothecoides*	Lipid (44.65%)	-	0.412	-	Krzemińska et al. (2019)
Municipal solid waste+ Cattle manure + Chicken manure	*Unfiltered* *TKN: 1.7 , NH_3-N: 0.96, COD: 12.6, TP: 0.1, TSS: 15.8*	10	*Chlorella* sp. and *Scenedesmus* sp. dominated mixed microalgae	Biomass	9-9,5	0,225	1.25	Ermiş and Altinbas, (2019)
Pig manure	*Unfiltered* NH_4^+ -N: 0.52, TN: 0.54, TP:0.21	100	*Chlorella* sp.	Biomass	8.28	-	1.1	Jiang et al., (2018)
Pig manure	*Centrifuged,* NH_4^+ -N: 0.81, *PO_4-P:0., COD:7.5*	8	*Chlorococcum* sp.	Biochemical composition	6.6	0.48	0.85	Montero et al., (2018)
Anaerobically digested effluent from kitchen waste (ADE-KW)	*Filtered 0.45μm, TN: 1.28; NH_3-N:1.18; COD: 6.1; TP: 0.011*	*6.7*	*Chlorella sorokiniana* SDEC-18 and *Scenedesmus* SDEC-8	Lipid (max. 48%)	8.31	0.098 and 0.118, respectively.	0.42 and 0.55, respectively	Zhang et al., (2018)
Vegetable Waste	*Centrifuged, NH_3-N:2 ; PO_4-P: 0.066, COD: 22.12*	*4 (Dosing time: 4 days)*	*Arthrospira maxima*	Biomass	7.5	0.2	0.12*	Massa et al., (2017)
Cattle manure	*Centrifuged, TN: 1.16; NH_3-N:0.91; COD: 6.2; TP: 0.061; PO_4-P: 0.041*	*13.19*	*Chlorella sp.*	Biomass	7	0.286	1.72	Zieliński et al., (2018)
Food waste and animal manure	*Unfiltered* *TN: 4 ; NH_3-N: 2.9; COD: 20; TP: 0.48; TSS: 3.26*	10	*Scenedesmus dimorphus*	Biomass	7.5	0.125	0.67	Hajar et al., (2017)

Table 1.2 (continued) : Microalgae species grown in anaerobic digestate.

Digester feed composition	Characterization of raw digestate (g L^{-1})	Dilution ratio (%)	Microalgae Species	Valuable Products	Working pH	μ (day^{-1})	Highest microalgae biomass (g L^{-1})	References
End-of-life dairy products with a mixture of agro-industrial wastes	*Filtered 0.7 µm, TKN: 1.75; NH_3-N:1.064; COD: 11.87; TP: 0.13; PO_4-P: 0.108*	10	*Parachlorella kessleri* and *Acutodesmus obliquus*	Biomass	9.7	-	1.07	Koutra et al., (2017)
Municipal and commercial food waste	*Filtered 10 µm, TN: 5.16; Ammonium: 3.19; TP: 0.136; PO_4-P: 0.071, TSS: 2.14*	25	*Nannochloropsis sp.*	Lipid (50%)	7.75	0.52	0.414	Mayers et al., (2017)
Pig slurry and corn	*Unfiltered TN: 3.35 , NH_3-N:2.05, COD: 17, PO_4-P: 0.32*	5	*Chlorella vulgaris*	Biomass	8	-	0.78	Franchino et al., (2016)
Swine Manure	*Unfiltered NH_3-N:0.058, PO_4-P: 0.095, TSS:0.12*	100	*Scenedesmus* sp.	Biomass	7	0.7	0.957	Dickinson et al., (2015)
Vinasse	*Unfiltered,* COD: 3.06, TN: 0.71, NH_4-N: 0.56, TSS: 1.3	10	*C. vulgaris* and nitrifying–denitrifying activated sludge	Biogas	7.84	-	0.6	Serejo et al., (2015)
Pig manure	*Filtered 1.2µ TN: 0.77 , NH_3-N:0.71, COD: 4, PO_4-P: 0.03*	5	*Desmodesmus sp.*	Biomass	7	-	0.385	Ji et al., (2015)
Livestock waste	*Settled. TN: 0.12 , COD: 3.2, TP: 0.13, SS: 0,3*	50	*Scenedesmus obliquus*	Biomass	6.4	0.382	0.311	Xu et al., (2015)
Food wastewater	*Centrifuged TN: 2.37 , COD: 5.9,* TP: 0.048	10	*Scenedesmus bijuga*	Lipid (max: 35%)	7.3	0.03	1.24	Shin et al., (2015)
Tetraselmis sp. effluent	*Unfiltered NH_3-N: 0.83, TP: 0.0074*	10	*Tetraselmis* sp.	Lipid (9%)	-	-	$0.5 \times 10^{6**}$	Erkelens et al., (2014)
Chroococcus sp.	*Unfiltered, TAN: 0.196, COD: 1.927*	30	*Chroococcus* sp.	Biogas	8.01	-	0.79	Prajapati et al., (2014)

Table 1.2 (continued) : Microalgae species grown in anaerobic digestate.

Digester feed composition	Characterization of raw digestate (g L^{-1})	Dilution ratio (%)	Microalgae Species	Valuable Products	Working pH	μ (day^{-1})	Highest microalgae biomass (g L^{-1})	References
Treatment sludge	*Unfiltered* *NH_3-N: 0.95, COD: 0.2, PO_4-P: 0.41, TSS: 1.13*	20	*Scenedesmus* sp. dominated mixed microalgae	Biomass	9-10	0.9	2.6***	Uggetti et al., (2014)
Municipal wastewate	*Centrifuged* *TN: 2.67 , COD: 2.66,* TP: 0.38	6	*Nannochloropsis salina*	Biomass	10	0.645	0.92	Cai et al., (2013)
Biogas effluent	*Filtered 0.7 µm,* *TN: 0.357, COD: 0.99, TP: 0.037*	16.6	*Chlorella* sp.	Biomass	6.77	-	0.615	Yan and Zheng, (2013)
Poultry litter	*Centrifuged* *TN: 1.57 , NH_3-N:1.11,* Phosphorus: 0.15	6	*Chlorella minutissima, Chlorella sorokiniana* and *Scenedesmus bijuga*	Biomass	-	-	0.612	Singh et al., (2011)
Dairy manure	*Screw-pressed,* *TN: 3, NH_3-N: 2.01*	2	*Neochloris oleoabundans*	Lipid (10%)	7.2	-	0.616	Levine et al., (2011)
Pig manure	*Filtered 1.2 µm,* *TN: 1.2, NH_3-N:1.12, COD: 1, TP: 0.07*	10	*Scenedesmus accuminatus*	Biomass	8,4	0.091	1.118	Park et al., (2010)

* g TSS L^{-1} d^{-1}
** cell mL^{-1}
***Dilutions performed results in TSS concentration at 1.8 g TSS L^{-1}

1.1.3 Microalgae and Biorefinery Concept

Circular bio-economy is an evolving perception, centering on the sustainable production, conversion, and utilization of renewable resources into value-added products. Photosynthetic organisms such as microalgae are the main goal in creating closed systems due to their eco-friendly and adaptable properties. Biorefinery approached combined with wastewater provides to manage economy and sustainability by resource recovery and by reducing the ecological footprint (Javed et al., 2019; Mohan et al., 2019a).

Microalgae produce a group of biochemical molecules, including carbohydrates, proteins, lipids, and nucleic acids, as well as essential vitamins and minerals. The cellular content of each fraction varies according to the specific strain of algae and their physiological responses to biotic and abiotic factors, e.g., light intensity, photoperiod, temperature, nutrients, and growth phase. With biorefinery approach, various and variable composition of microalgae are seen as biotechnological potentials. Not only the lipids for biodiesel production is produced by microalgae, but valuable by-products can also be obtained from them. Therefore, in biorefinery processes microalgae are seen as most suitable candidate (Peralta-Ruiz et al., 2013).

Microalgae grown on wastewater present an enormous potential as biofertilizer. The ability of microalgae to uptake nutrients such as C, N and P from wastewater results in an enhancement of nutrient availability for plant systems.Microalgae present a chemical composition, including macronutrients such as N, P and K , better than available organic fertilizers (Mahapatra et al. 2018). Eventhough microalgae area source of proteins, lipids and carbohydrates,there are few studies regarding microalgae supplementation to animal diets. Microalgae biomass grown in wastewater is generally characterized by high-protein contents, so that a possible valorization way is its use as protein source for animal feed. For instance, microalgae were included in rainbow trout diets in percentages of 12.5%, 25% and 50%. The results evidenced that an inclusion higher than 12.5% resulted in nutritional deficiencies in trout (Dallaire et al. 2007).

Algae can accumulate 30-80 % of lipids which normally consist of 90-95% of triacylglycerides. Most of the algal strains such as *Chlorella* produce FA ranging from C16-C18 which are suitable for biodiesel production; similar in properties to traditional fossil-based diesel. Several microalgae species have a tendency to produce

health related FA like omega-3, omega-6 and docosahexaenoic acid (DHA) as major algal-based FA. They are non-toxic and more stable as compared to fish DHA (Kumar and Singh, 2019). Cyanobacteria and algae are the natural sources for carotenoids and diverse carotenoids including astaxanthin and lutein. Production of these compounds can be improved through abiotic factor manipulation. Wastewater grown algae can be utilized for this purpose and provides a cost-effective sustainable alternative. Astaxanthin has vast applications in medical sciences due to its anti-oxidant, anti-inflammatory, anti-cancer, and anti-aging properties. Moreover, it also improves the nervous system, respiratory system, fertility, and digestive system. Wastewater integrated algal-growth has been suggested for improved astaxanthin production. Similarly, other carotenoids have shown various nutraceutical properties. Cyanobacteria (group of algae) produce large amounts of industrially and nutraceutically important compounds called phycobilins (Pancha et al., 2019) which are colored protein-pigment complexes having antimicrobial, anticancer, and antioxidant properties. They can also be used to produce proteinrich energy drinks and can be applied has food colors.

Wastewater-cultivated algae could be used as animal feed, biofertilizer, and cosmetic agents. Biochar produced by algal pyrolysis is rich source of nutrients and can be utilized as biofertilizer in agriculture. *Spirulina, Chlorella, Euglena, Tetraselmis, Synechococcus, Nannochloropsis* have been utilized for human consumption as well as aquaculture and animal feed supplement due to their high protein content and nutritional value (Shahid et al., 2020). Algal extracts have been widely used in cosmetic industry as skin and hair protectants due to their anti-oxidant, anti-irritant, anti-aging, sun-protecting and tissue regenerating abilities. *Chondrus, Chlorella, Dunaliella, Nannochloropsis, Spirulina* have been widely utilized for commercial cosmetic products (Javed et al., 2019; Shahid et al., 2020).

In conclusion, microalgae biomass can be used for different applications. These applications include energy production (i.e. biofuels), products for agriculture (biofertilizers, biopesticides etc.), animal feed and products for human consumption (foods and pharmaceuticals). When biomass is obtained as a by-product after wastewater treatment, low-cost applications are preferred, since only microalgae recognized as safe (GRAS) can be sold for human consumption. Therefore, the most common uses are energy, biofertilizer and animal food production.

1.2 Problem Statement

During anaerobic digestion, organic nitrogen is converted to ammonia nitrogen and total phosphorus to orthophosphate. For this reason, high concentrations of ammonia and phosphorus are present in the anaerobic digestion effluent . Themost common method for decaying effluent is direct application to land. Direct disposal of the digestive effluent is considered a cheaper method in terms of fertilizer use (mineral and organic matter) for agricultural areas. Especially high content of organic matter is a desirable condition in some agricultural fields (Grigatti et al., 2011; Chadwick, 2007; Schievano et al., 2009). However, a decomposing effluent which is not fully consumed in terms of readily decomposable organic compounds can also be obtained. This can lead to problems in the storage stage (odor emission, regeneration of pathogens and phytotoxicity) and negative effects on the soil-plant system by limiting the potential fertilizer value. These can lead to damaging effects on large scale plants, such as delays in planting seeds, plant mortality, or growing stresses.Recently, microalgae have been applied in nutrients recovery from AD digestate. The use of microalgae in digestate treatment has a number of benefits: high growth and nutrient uptake rates, large fertilizer need, carbon capture and biomass production. Besides, high growth rates of microalgae can significantly lower land area for digestate application. Additionally, there is a possibility of sourcing CO_2 from biogas as carbon source for microalgae cultivation, which can facilitate simultaneous biogas upgradation and digestate treatment. However, microalgae cultivation in digestate requires alleviation of ammonia toxicity, which may be harmful for the microorganisms at high concentrations. Moreover, the presence of readily biodegradable organic compounds makes digestate vulnerable to bacterial invasion, and the resulting turbidity may reduce photosynthetic efficiency, nutrients removal and biomass productivity. Additional limitations may arise from a relatively low phosphorus to nitrogen (P/N) ratio in the digestate, which may not fulfil the stoichiometric P demand of microalgae.

1.3 Purpose of Thesis

Algal biomass has several benefits and is a sustainable source. Food, pharmaceutical, biodiesel, and biogas biomass are just some of the beneficial uses of the algae. Beside those, it has a significant contribution to the global carbon cycle by acting as a principal consumer. Also, one of the unique properties is the rapid growth compared to other

plants. However, they need high amount of nutrients. In this concept, wastewater can be a attractive and promising solution as being raw material for microalgae. The nutrients present in the wastewater is accepted as pollutants and have to be treated before discharging through the water bodies. Coupling the concepts of wastewater treatment and microalgae growth is a sustainable solution by providing economical efficiency and protection of human health. The purpose of this thesis were to show the applicability of recovery of nutrients from wastewater, instead of treating with advanced treatment methods and to recover high value products from the algal biomass cultivated in wastewater to lower the cost of production of microalgae and to apply the biorefinery appoach.

1.4 Hypothesis

Recycling nutrients from digestate with algal technology is at an early stage. Coupling of microalgae growth with liquid digestate treatment is an economical and ecologically friendly technology for cheap biomass production for further valuable applications. In microalgal technology, among the numerous types of wastewater which have the potential of displacing synthetic nutrients, digestates may play a key role; where several strategies should be adopted to overcome the obstacles in microalgal growth associated with digestate turbidity, ammonia toxicity, or phosphorus limitation. In this aspect, this chapter will discuss challenges and opportunities associated with developing of this new technology and provide alternative strategies towards the cultivation of mixed microalgae consortia, which could be further converted in to biofuels and value added chemicals with a biorefinery understanding along with the digestate treatment.

Amongst the many thousands of microalgal species present in nature, there are only a few commonly occurring species currently studied and known to be robust survivors in wastewater or in digestate. Both freshwater microalgae (*Scenedesmus* sp. and *Chlorella* sp.) and marine microalgae (*Nannochloris* sp.) have effectively removed nutrients from liquid digestate. In a similar way, *Dunaliella* sp., a microalga adapted to extreme environmental conditions, has also proven suitable for nutrient removal in highly concentrated liquid digestates. These include species belonging to the genera *Chlorella, Scenedesmus, and Desmodesmus* with key species being *Chlorella vulgaris* and *Scenedesmus obliquus* here the elemental characteristics of digestate can meet the

nutrient requirements of microalgae during the cultivation, and thus the digestate can serve as a nutrient-rich, organic fertilizer for the production of microalgae. However there is no study on mixed culture which has many advantages comparing to pure culture such as adapting the harsh conditions easly.

In the environmental field, coupling microalgae growth with wastewater treatment appears to be a promising solution to overcome current high costs of microalgae cultivation and, at the same time, to solve specific treatment problem such as the compliance to standards of nutrient concentration in effluents. With this regard, agriculture wastes appear to be good candidates and, among others, the digestate obtained through the anaerobic digestion process is particularly promising for its high content of mineralized nutrients. Microalgal cultivation in digestates represents a promising strategy for simultaneous wastewater treatment and valuable biomass production.

Recycling the nutrients from AD and assimilating them into algal biomass can result in further feedstock for the process without incurring the monetary or environmental costs of using nitrogenous or phosphorus fertilizers while simultaneously remediating the liquid waste stream from the process.

2. DETERMINATION OF BIOKINETIC COEFFICIENTS FOR NUTRIENT REMOVAL FROM ANAEROBIC LIQUID DIGESTATE BY MIXED MICROALGAE[1]

2.1 Introduction

Anaerobic digestion is a simple natural prosess for converting waste into energy. Several environmental benefits can be achieved with anaerobic digestion such as renewable energy generation, methane emissions reduction and manure management. On the contrary, characteristics of the digestion effluent, such as some undesirable odor, viscosity, high humidity and high volatile fatty acids, can cause phytotoxic effects in plants (Walker et al., 2009). Moreover, untreated anaerobic liquid digestate may cause eutrophication if directly discharges into the water bodies. For this reason, the application of agricultural land without treatment may constitute a risk (Abdullahi et al., 2008). Therefore solutions for targeting both reducing adverse effect and increasing product values by valorization should be followed. Microalgal technology is one of the promising approaches to remove nutrients from wastewater by assimilating nitrogen and phosphate into their biomass. They show higher efficiency in nutrient removal than other microorganisms since the nutrients present in various wastewaters are essential for microalgal growth (Salama et al., 2017), while fixing CO2 through photosynthesis. Numerous scientific studies are underway to reduce the cost of algal biomass production so that the use of biomass for energy production becomes feasible. The important factors contributing to the cost of algal biomass production include the nitrogen and phosphorus supplements required for algal growth. Therefore, it would be advantageous to obtain these nutrients from the wastewater in order to reduce the costs of the produced biomass.

[1]This chapter is based on the paper: Ermis, H., & Altinbas, M. (2019). Determination of biokinetic coefficients for nutrient removal from anaerobic liquid digestate by mixed microalgae. Journal of Applied Phycology, 31(3), 1773-1781.

Coupling of microalgae growth with liquid digestate treatment is an economical and ecologically friendly technology for cheap biomass production for further valuable applications such as food, feed, fuel, fertilizers, and fine chemicals. This technology helps to reduce the effluent's pollution load by microalgae growth and become a sustainable and a promising solution.

The nutrient removal kinetic from wastewater is the key factor for determination of system design and performance because microalgae grown in various digestate indicate that nutrient removal and biomass production potentials depend on the microalgal species and the characteristics of the digestate. There was a lack of data in the literature not only nutrient removal kinetics of microalgae on wastewater, but also spesifically removal kinetics of mixed microalgae on anaerobic liquid digestate where this study filled this gap of information.

The aim of this study is to examine the nutrient removal performance of mixed microalgae at different dilution ratios of anaerobic liquid digestate in batch operation and to determine biokinetic coefficients for ammonia and orthophosphate. A study of the kinetics of nutrient removal via microalgal growth is fundamental for microalgae-based wastewater treatment performance estimation for examining the optimum growth conditions for the spesific wastewater. Even though there are previous studies conducted with microalgae growth on anaerobic liquid digestate, those studies were focused on mainly with pure/defined cultures and giving only μ values lacking of nutrient removal kinetics. Therefore, to the best of the authors knowledge, there are no study that examines the kinetic coefficients of mixed microalgal growth on anaerobic liquid digestate.

2.2 Materials and Methods

2.2.1 Feedstock characterization

In this study, the Anaerobic Liquid Digestate (ALD) obtained from full scale plant which is decomposing of the waste mixture given in Table 2-1.

Table 2.1 : Ingredients of Anaerobic Digester Influent.

	Weight (t)	Weight (%)	TS (%)	TS (t)
Mechanically/manually separated organic fraction of municipal solid waste	60	50	7	4.2
Cattle manure	20	17	8	1.6
Leaching water from solid waste collection vehicles	10	8	5	0.5
Expired market wastes	5	4	10	0.5
Chicken manure	5	4	65	3.25
Water	20	17	7	1.4
Total	120		10	11.45

After anaerobic digestion, this dark ALD (46,666 ± 6005 Pt-Co) was used as a growth medium for microalgae cultivation. According to the characaterization of ALD used in this experiment: COD (mg L^{-1}) was 12.,600±300, TKN (mgL^{-1}) was 1692 ± 256, NH_3-N (mg L^{-1}) was 900 ± 62, NO_3-N (mg L^{-1}) was 0.13±0.02, TP (mg L^{-1}) was 105 ± 7.5, PO_4-P (mg L^{-1}) was 64 ± 6, TSS (mg L^{-1}) was 15,880 ± 932, and pH was 9.00-9.15, which are consistent with the values in the literature (Alburquerque et al., 2012; Haraldsen et al., 2011; Magrí et al., 2013).

2.2.2 Preparation of microalgal inoculum

A mixture of several spontaneously reproducing indigenous species isolated from local ponds nearby Istanbul Technical University,Istanbul, Turkey and inoculated in diluted ALD (2%) for acclimation. The inoculation had been cultivated in 5% ALD for five generations to obtain stable characteristics. Cultures were kept in an acclimation cabinet under approximately 150 µmol photon m^{-2} s^{-1} continuous illumination measured with a light meter (Hansatech QRT1 Quantitherm), at 25 °C ± 2 °C during the acclimation period. Aeration was provided by shaker used at 130 rpm and also to prevent cells sedimentation while keeping the batch system in completely mixed conditions.

Inoculum cell culture was examined by light microscopy and mixed culture was morphologically characterized by using microalgae systematics books (Barsanti and Gualtieri, 2014; Pröschold and Leliaert, 2007; Bellinger and Sigee, 2015). According to the microsobic observation, the mixed culture clearly dominated by *Chlorella* sp. and *Scenedesmus* sp.

2.2.3 Experimental set-up

The experiments were conducted in batch mode by using 1000 mL erlenmayer flasks in triplicate with 750 mL working volume with no aeration. Lower dilution ratios (2%, 5%, 7% and 10%) were used in this study because of the low growth rates observed in higher dilution ratios more than 10% (Abu Hajar et al., 2016, 2017; Bjornsson et al., 2013; Bohutskyi et al., 2015; Cai et al., 2013; Franchino et al., 2013; Koutra et al., 2017; Ledda et al., 2016; J. Park et al., 2010; Prajapati et al., 2014; L. Wang et al., 2010; Zhao et al., 2014). Moreover, faster growth in a shorter time with more accurate measurement were observed with lower dilution ratios. The influent NH_3-N concentration was varied between 18.6–90.1 mg L^{-1} while influent PO_4-P concentration was between 1.85–6.88 mg L^{-1}. The experiments were performed in an acclimation cabinet under approximately 150 µmol photon m^{-2} s^{-1} continuous illumination at 25 °C ± 2 °C. All batch systems were started with 4 mg L^{-1} chlorophyll-a. The chlorophyll a (Chl a) were followed daily to observe microalgae growth. Dry weight, NH_3-N, TKN, TP and PO_4-P were measured at the beginning and the end of the batch systems. In all batch systems, the initial algae biomass concentration were started 0.14 g L^{-1}.

2.2.4 Analytical methods

Nitrogen and phosphorus were analysed as major nutrients for microalgal growth. Nitrogen was measured as Total Kjeldahl Nitrogen (TKN) and Ammonium (NH_3-N); Phosphorus was measured as Total Phosphorus (TP) and Orthophosphate (PO_4). Since microalgae prefer ammonium as a nitrogen source comparing to all other nitrogen sources (González-Camejo et al., 2018), NH_3-N was followed throughout the study. Suspended Solid (SS), TKN, NH_3-N, TP and PO_4-P values analysed as mg L^{-1} according to the Standard Methods (Rice, E.W. Baird, R.B. Eaton, 2017).

To determine the Chl a content, 2 mL of algal suspension was centrifuged at 3000 rpm for 10 minutes and the supernatant was discarded. The algae were suspended in 0.6 mL of methanol and heated to 80 ^oC for about 5 minutes in a water bath. The samples were cooled to room temperature and then the volume was made up to 1 mL by adding methanol. The Chla concentration was calculated with a spectrophotometer at the given wavelength below against a solvent blank by using the equation below (Aslan & Kapdan, 2006):

$$\text{Chlorophyll a (mg L}^{1}) = (16.5\times A_{665})-(8.3\times A_{650}) \quad (2.1)$$

2.2.5 Kinetic coefficient calculations

The microalgal growth was monitored by measuring the total chlorophyll concentration (Chl; mg L^{-1}) during the experiment due to the dark color of wastewater. A recent study by (Marazzi et al., (2017) and Huy et al. (2018) reported that measuring OD values for liquid digestate could be a challenging due to their strong color. Nutrient removal kinetics were also monitored by measuing the chlorophyll, because chlorophyll synthesis affected by the availability of nitrogen and nitrogen is a structural element of chlorophyll and protein molecules, and therefore affects the formation of chloroplasts and accumulation of chlorophyll in microalgae (Adesanya et al., 2014). The specific growth rate (μ) of microalgae were calculated using Eq. Below (Aslan & Kapdan, 2006; Delgadillo-Mirquez et al., 2016):

$$\mu = \frac{lnChli - lnChl0}{ti - t0} \quad (2.2)$$

Where Chl_t and Chl_i are the chlorophyll concentrations at the times t_0 and t_i corresponding to beggining and end of the exponential growth phase, respectively.

The time required to double the population, doubling time (t_d) was calculated from the value of the specific growth rate (Delgadillo-Mirquez et al., 2016):

$$td = \frac{ln2}{\mu} \quad (2.3)$$

The removal rates (R_i) were calculated according to Eq. below:

$$Ri = \frac{So - Sf}{ti - t0} \quad (2.4)$$

Where R_i represents the nutrient removal rate of the substrate (NH_3-N or PO_4-P) at t_i.

Removal efficiency was calculated with the following equation:

$$\text{Removal Efficiency} = \frac{(So - S) * 100}{So} \quad (2.5)$$

The specific rate of substrate removal (R_{xi}) were calculated according to Eq. below:

$$Rxi = \frac{Ri}{Chl0} \tag{2.5}$$

Experimental data were plotted in form of $1/Rx_i$ against $1/S_0$. The Michaelis-Menten kinetic relitionship was used in determination of kinetic coefficients K_m (saturation constant) and k (reaction rate constant) from the slope and intercept of best fit line of this plot were determined. Michaelis-Menten kinetic equations were employed for the determination of two kinetic coefficients because this equation is suitable for the data since the rateof substrate utilization gets higher with high organic content and vice versa (Saidu et al., 2017).

Yield coefficient for NH_3-N and PO_4-P removal was calculated by using eq. below (Aslan & Kapdan, 2006):

$$Y_N = \frac{Chlf - Chl0}{NH40 - NH4f} \tag{2.6}$$

$$\text{Yp} = \frac{Chlf - Chl0}{PO40 - PO4f} \tag{2.7}$$

Mass balance was performed to confirm the correlation between nutrient removal and biomass yield. Stoichiometric equation was used to calculate the theoretical microalgae formation (Ebeling et al., 2006) which corresponds to 6942 g mol^{-1}. Afterwards, the experimental results of microalgal biomass were compared to the theoritical calculations.

$$16\,NH_4^+ + 92\,CO_2 + 92\,H_2O + 14\,HCO_3^- + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 106\,O_2 \tag{2.8}$$

2.2.6 Free ammonia calculation

Free ammonia was calculated according to the following equation with by taking K_a (25 ^oC) constant as $5{,}75e^{-10}$. Therefore, pH-dependent free-ammonia loss was calculated for the sets for all dilution ratios. This calculation was made for the ammonia nitrogen measurement on the last day of the all batch sytems.

$$[NH3] = \frac{TAN}{1 + [\mathrm{H}]/Ka} \quad (2.9)$$

TAN: Total ammonium nitrogen

K_a: The acid balance constant for ammonium

2.3 Results

2.3.1 Biokinetic coefficient determination

The Michaelis-Menten Kinetics was used to calculate the half saturation constant (K_m) and the reaction rate coefficient (k). Triple sets with dilution ratios of 2%, 5%, 7% and 10% were established for kinetic calculations. The plot of $1/Rx_i$ against $1/S_o$ gives linear line consisting of slope = K_m/k and intercept at y-axis = 1/k (Figure 2.1). The kinetic constants for the NH_3-N removal over these dilution ratios were determined to be reaction rate (k) is 2.48 mg NH_3-N mg^{-1} chl a day^{-1} and half-saturation constant (K_m) 29.3 mg L^{-1}. The kinetic constants calculated for PO_4-P removal were determined to be k: 0.21 mg PO_4-P mg^{-1} chl a day^1 and a half saturation constant (K_m) of 2.94 mg L^{-1}.

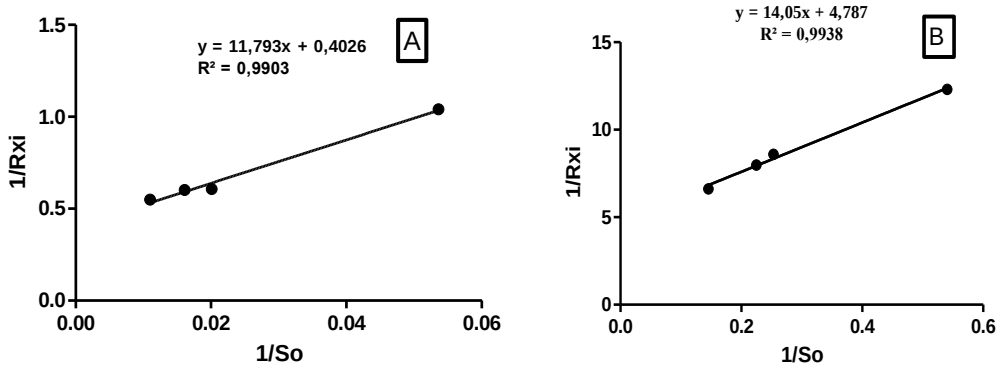

Figure 2.1 : (A) $1/S_o$ - $1/R_{xi}$ graph of NH_3-N, (B) 1/So - $1/R_{xi}$ graph of PO_4-P.

2.3.2 Nutrient uptake effect on algal yield and growth rate

Algal yield (Y) was obtained by plotting a graph of chlorophyll difference against substrate difference on the first day and the last day, and the slope of the graph obtained gives the yield coefficients (Figure 2.2). In this study based on Michaelis-Menten Kinetics, the yield coefficient (Y_N) for nitrogen was 0.48 mg chl a mg^{-1} NH_3-N (R^2 =

0.97) and the yield coefficient (Y_P) for phosphorus was found to be 5.72 mg chl a mg^{-1} PO_4-P (R^2 = 0.95). Experimental results indicated that for the same amount of chlorophyll formation, nitrogen is used 10 times less than phosphorus which makes nitrogen 10 times more preferable.

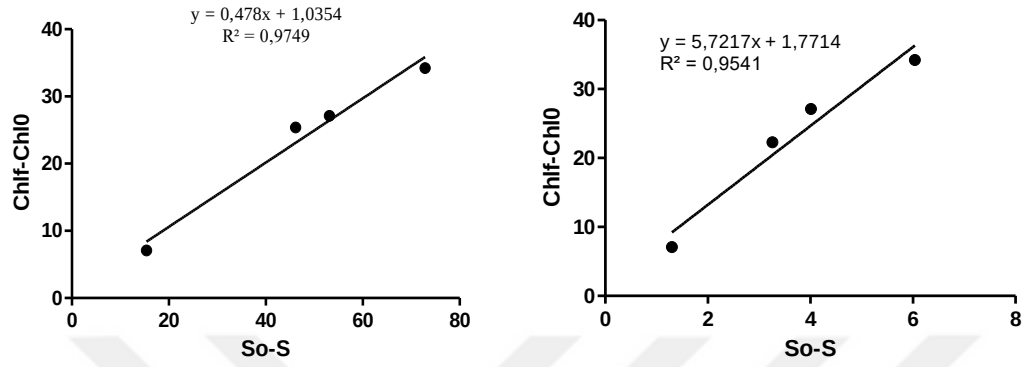

Figure 2.2 : Y_N and Y_P , respectively.

The highest chlorophyll production was observed at the 10% dilution rate (29.8 mg L^{-1} or 3.31 mg $L^{-1}d^{-1}$) in which the highest nutrient was available. When the nutrient value was the lowest (2% dilution ratio), the shortest doubling time with the highest growth rate was observed with 0.32 d^{-1}. However, microalgae was passing to the death phase without reaching the highest amount of chlorophyll (11 mg L^{-1}) as high as 10% dilution ratio (29.8 mg L^{-1}) with lower NH_3-N removal (71%) comparing to 10% dilution ratio (90%).

For both NH_3-N and PO_4-P,the amount of removed nutrient tended to increase with high initial ALD concentration. Therefore, highest removal rate were observed at 10% dilution ratio for both nutrients (Table 2-2).

Table 2.2 : Growth rate and doubling time of microalgae for different dilution rates.

Dilution (%)	Specific growth rate,μ, day^{-1}	T_d, Day	Operation Time, Day	Biomass (TSS), g L^{-1}	Peak Chl. amount, mg L^{-1}	Removal Rate (mg NH_4-N L^{-1} day^{-1}	Removal Rate (mg PO_4-P L^{-1} day^{-1}
2	0.32	2.18	4	0.42	11.1	3.83	0.27
5	0.29	2.39	7	0.76	29.38	5.83	0.47
7	0.25	2.81	8	0.87	31.1	5.71	0.51
10	0.22	3.08	10	1.24	38.2	7.05	0.60

The removal rates achieved from this study was greater than most studies (Figure 2-.3) where Gao et al. (2016) examined 0.59 mg N L^{-1} d^{-1} and 0.08 mg P L^{-1} d^{-1} when

Chlorella sp. were cultivated in real treated sewage. As in previous dilution studies done with 5%, 10%, 20%, 40%, 60%, 80% and 100% dilution ratios by authors (data not shown), kinetic studies have also shown that the most efficient system was 10% dilution ratio.

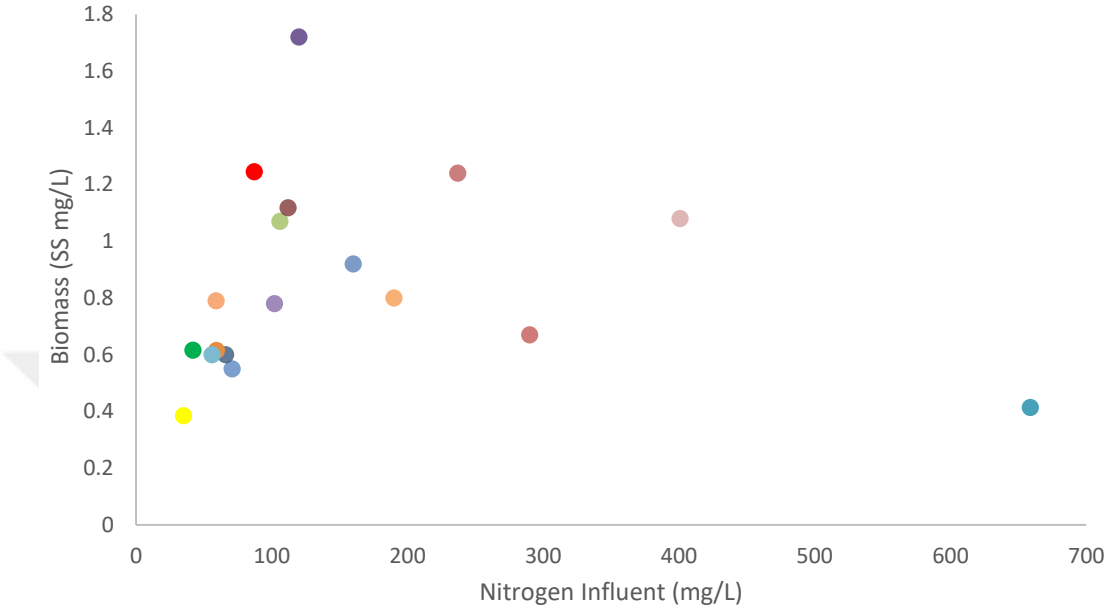

Figure 2.3 : Correlation between nitrogen influent and algal biomass (This study is shown in red) (Zhang et al., (2018): 0.55 g L^{-1}, Massa et al., (2017): 1.08 g L^{-1}, Zieliński et al., (2017): 1.72 g L^{-1}, Hajar et al., (2017): 0.67 g L^{-1}, Koutra et al., (2017): 1.07 g L^{-1}, Mayers et al., (2017): 1.41 g L^{-1}, Franchino et al., (2016): 0.78 g L^{-1}, Serejo et al., (2015): 0.6 g L^{-1}, Ji et al., (2015) 0.38 g L^{-1}, Shin et al., (2015): 1.24 g L^{-1}, Prajapati et al., (2014): 0.79 g L^{-1}, Uggetti et al., (2014): 0.8 g L^{-1}, Cai et al., (2013): 0.92 g L^{-1}, Yan and Zheng, (2013): 0.61 g L^{-1}, Singh et al., (2011): 0.6 g L^{-1}, Levine et al., (2011): 0.62 g L^{-1}, Park et al., (2010):1.12 gL^{-1}).

Moreover, higher growth productivity was also observed at 10% dilution ratio (0.12 g L^{-1} d^{-1}). This productivity was also higher comparing to other studies with pure culture cultivated in liquid digestate (Cai et al., 2013; F. Ji et al., 2015; Levine et al., 2011; J. Park et al., 2010; Singh et al., 2011) because mixed microalgae can be able to perform better than pure culture by changing species dominance due to stres conditions caused by wastewater.

2.3.3 Mass balance

While mass balance is being established, free-ammonia amount due to high pH leaving the system has been taken into consideration in order to ensure to obtain correct results. It was observed that pH was reached to over 9 for all the batch systems.The calculated

ammonia concentration in the form of free-ammonia nitrogen form was theoretically calculated 2.4, 13.3, 13.9 and 31.8 mg L^{-1} for 2%, 5%, 7%, and 10% dilution ratio, respectively. For this reason, when microalgae growth is observed, free-NH_3-N due to pH change should be included as a factor in calculations. NH_3-N removal in the calculation of nitrogen removal effects on the positive direction, indicating that the removal rate is higher than it is.

90% NH_3-N removal was observed in the system running for 10 days with 10% dilution ratio. However, not all removed nitrogen contributed to microalgae synthesis. To figure out how much nitrogen contributed to biomass synthesis to free ammonia nitrogen concentration was calculated considering pH. Afterwards, this amount was taken into account for the estimation of correct biomass synthesis (Table 2.3).

Table 2.3 : Mass balance based on ammonia nitrogen**.**

Dilution ratio (%)	2%	5%	7%	10%
Experimenta observed biomass (mg VSS)	151	295	338	467
Calculated free ammonia according to Eq.10 (mg L^{-1})*	2.4	13.3	13.9	31.8
Experimental total ammonia removal, (mg L^{-1})**	13.4	39.2	47.1	76.8
Calculated total ammonia consumed by microalgae, (mg L^{-1})	11	25.9	33.2	45.0
Theorical biomass (mg VSS) according to total ammonia consumed by microalgae (Eq. 9)	102	240	307	416
Unknown biomass (mg VSS)	49	55	31	51

* Assumed all free nitrogen relesed from the system.

** Subtraction of last day of ammonia from first day of ammonia

Microalgal mass balance calculations were performed according to equation 9. Experimentally the total consumed NH_4^+-N concentration including NH_3-N released via pH change with a working volume of 0.75 L was 151, 295, 338, and 467 mg VSS for 2%, 5%, 7%, and 10% dilution ratio, respectively. According to the experimental NH_3-N after subtracted the calculated free ammonia amount, the theorical biomass according to the total NH_3-N consumed by microalgae was 102, 240, 307 and 416 mg for 2%, 5%, 7%, and 10% dilution ratio, respectively.

2.4 Discussion

2.4.1 Biokinetic coefficients

According to the results, it was observed that PO_4-P was above the saturation throughout all experiments. These results were in line with Aslan & Kapdan (2006)'s study which were k_N = 1.5 mg NH_3-N mg^{-1} chl a d^{-1}, Km_N = 31.5 mg L^{-1} for nitrogen and k_P = 0.5 mg PO4-P mg^{-1} chl a d^{-1}, Km_P = 10.5 mg L^{-1} for phosphorus, eventhough it was a study on synthetic wastewater. Wang et al. (2014) also studied the nutrient kinetics of *Chlorella* sp. on the primary effluent and sludge centrate from the dewatering process of anaerobically digested sludge of local wastewater treatment plant and according to their study, P removal by *Chlorella*sp. were determined to be Km: 3.01 mg L^{-1} (R2 = 0.87) which is in line with this study.

The implication of these results was that the K_m values represent the maximum amount of the nutrients that mixed microalgae can assimilate for growth if the system were to be in continuous mode. Therefore, those results established an affinity to PO_4-P. The Michaelis constant, K_m, is defined as the substrate concentration at which the reaction rate is half of the maximum,V_{max}. Therefore with a high K_m, high amount of substrate is needed to reach $V_{max}/2$; with a low K_m, there is no necessity to substrate in the solution to reach $V_{max}/2$. Hence, the higher K_m means the lower the affinity for substrate. Aslan and Kapdan (2006) reported also high affinity to PO_4-P in their study. The high k value usually favors high biodegradation reaction of an organic substrate The rate of nutrients removal (k) values obtained from this study confirmed that mixed microalgae can provide high removal of these nutrients. The R values for the determination of these coefficients are above 99; this indicates that the kinetic coefficients computed from this experiment are accurate.

In spite of the fact that, mostly studies conducted their experiment with filtered ALD, this study gives a opportunity to work with real scale with a high microalgal growth (1.25 g L^{-1}). However, further studies on ALD especially for mixed microalgal culture should continue to examine the higher growth stimulation factor for mixed microalgae.

2.4.2 Microalgae growth and nutrient removal

The results of specific growth rate (μ) and doubling time (t_d) were calculated according to the results of chlorophyll-a concentrations, since microalgae growth was monitored

by measuring chlorophyll-a concentration (chlorophyll-a mg L^{-1}) (Delgadillo-Mirquez, 2016; Aslan and Kapdan, 2006). However, last date of the experiments, SS and VSS analyses were followed for the determinaton of the amount of the algal biomass. The growth rates obtained from this study (0.22-0.32 d^{-1}) was in the range of the the growth rate observed in other studies examined on anaerobic liquid digestate (Ayre et al., 2017; Bohutskyi et al., 2016; Cheng et al., 2015; Mayers et al., 2017; J. Park et al., 2010; Uggetti et al., 2014; Xia & Murphy, 2016).

Similar other studies by Huy et al. (2018), Wang et al. (2010) and Salgueiro et al. (2016) achieved growth rate of 0.17 d^{-1}, 0.28 d^{-1}, 0.4 d^{-1} of cultivated *Chlorella* sp. on anaerobic digested, respectively. Other authors found similar growth rates of either *Chlorella* or *Scenedesmus* in livestock manure between 0.31 and 1.2 d^{-1} (Kumar et al., 2010; Travieso et al., 2008).

Uggetti (2014) mentioned that literature values for microalgal growth rate on wastewaters other than anaerobic liquid digeaste are between 0.2 and 1 d^{-1}. Moreover, in Kalana (2015)'s study, the highest total specific growth rate (μ) was found to be 0.7 d^{-1} when *Chlorella vulgaris* was grown in synthetic wastewater with a maximum biomass concentration 0.79 g L^{-1} in a continuous process. This reveal that anaerobic liquid digestate can compete with other wastewaters despite its dark color and highly particulate rich matter.

A detailed literature search on microalgae grown in anaerobic liquid digestate was plotted in form of nitrogen influent against maximum algal biomass in Figure 2.3. Eventhough the range of dilution ratio and digestate composition vary dramatically, it can be seen in Figure 2.3 that there is a correlation between influent nitrogen and microalgal biomass. According to the Figure 2.3, 90% of the studies conducted with anaerobic liquid digestate was under 290 mg N L^{-1}. Ward et al. (2014) mentioned that minimum concentration of 50 to 200 mg N L^{-1} of nitrogen as ammonia is essential for the requirements of the microalgal community which support Figure 2.3.

The lower dilutions caused lower chlorophyll a formation which was the reason of linkage of chlorophyll synthesis to nitrogen uptake where low nitrogen availability leads to less chlorophyll production in microalgae (Adesanya et al., 2014). Eventhough the main aim of this study was not wastewater treatment, TKN and TP were also measured to be able to comment about water quality requirements. According to the

results, strong wastewater characteristics of ALD dropped to the level of domestic wastewater quality in terms of TKN and TP which were ranging between 14.7-88.1 and 0.35-3.4, respectively; where be directly discharged to the sewerage system.

To the best of the authors knowledge, the highest biomass was obtained from this study (1.25 g L^{-1}), except Zieliński et al., (2017)'s study which was 1.72 g L^{-1} (Figure 2.3). The reason Zieliński et al.(2017) achieved higher biomass than this study can be the reason of working with neutral pH (7) which may have positive effect on microalgae growth compared to higher pH observed in this study.

As it is observed from Fig. 2.3, there is an algal biomass increase until around 100 mg N L^{-1} followed by fixed biomass amount despite of increasing influent nitrogen concentration. This stable line shows existence of limiting factor of microalgal biomass amount. However, this case may also depend on other factors such as color of wastewater or particule matter, independently from nitrogen.

Since this study is focused on the growth kinetic of the mixed microalgae culture on anaerobic digestate, the cost efficiency was not proven. However, Xin et al., (2016) mentioned that microalgae cultivation based on anaerobic digestate significantly reduced the biomass production cost of which had been estimated to be about $0.33/kg. Therefore, wastewater based algae production can not only save nutrients cost (around $550,000/yr), but also save anaerobic digestate treatment cost around $564,768/yr (Xin et al., 2016). However some studies applied autoclave to sterilize ALD for microalgae cultivation which is not feasible in term of time-wise and cost (Levine et al., 2011; Massa et al., 2017; Mayers et al., 2017; J. Park et al., 2010; Shin et al., 2015; Zieliński et al., 2017).

2.4.3 Mass balance

According to the mass balance, it was assumed that there was no nitrification since nitrifying bacteria are very sensitive microorganisms where Nitrosomonas and Nitrobacter has an optimal pH between approximately 7.0 and 8.0 (*EPA,2002*) which is also supported by Skadsen et al. (1996) who reported that a greater pH than 9 can be used to reduce the occurrence of nitrification. Since ALD original pH is 9 and the system reached pH 10.5; it was assumed that there was no nitrification in the system.

It was also assumed that the amount of NH_3-N after subtracted the calculated free ammonia was all consumed by microalgae, not by bacteria according to the no

presence of bacteria was observed microscopically due to high pH in all batch systems. It is considered that the reason of the amount of biomass obtained higher than the theoretical calculation which is called "unknown biomass" in **Table 2.3** was possibly derived from the particulate matter present in wastewater.

2.5 Conclusions

In this study, locally isolated wild type mixed microalgae were cultivated in differently diluted ALD to identify the optimal algal growth rate and nutrient removal kinetics. Based on the experimental data for biokinetic coefficients, mixed microalgae grown in anaerobic digestate had an affinity to PO_4-P while Y_N was 10 times higher than Y_P which made NH_3-N more preferable. This study demonstrated that ALD can be used as the nutrient rich cultivation medium for mixed microalgae culture with high growth rates, despite the fact that liquid digestate is not the ideal growth media for microalgae due to its high ammonia content, dark color, and particulate matter. Working with mixed microalgal culture reduces the risk of system contamination compared to working with pure culture which generates the operation in difficult conditions easier by changing its dominance and increase the process feasibility.

3. EFFECT OF SALINITY ON MIXED MICROALGAE GROWN IN ANAEROBIC LIQUID DIGESTATE[2]

3.1 Introduction

Rapidly developing industrialization as a result of the ever-increasing demands of the world's population and its associated needs has created a serious energy shortage in the last century (Gilpin, 2018). One of the research areas that has been emphasized in recent years is biofuels that can be obtained from biomass and various sources of biomass. Even though biomass energy can be obtained from terrestrial plants and water plants, research in recent years has shown that the production of microalgae is advantageous in many aspects compared to terrestrial plants (Mata et al., 2010; Peng et al., 2018; Shuba & Kifle, 2018).

Microalgae is a sustainable source for food, feed, and/or biofuel with a significant contribution to the global carbon cycle by acting as a principal consumer. By not requiring arable land, their higher productivity with seasonless rapid growth, and higher biofuel yield compared to terrestrial plants, it drew the highest attention in the field of biodiesel comparing to the other conventional biomass sources. Lipids are usually accumulated in the microalgae cells under undesirable environmental or stress conditions by altering their lipid biosynthetic pathways toward the neutral lipids formation (T. Wang et al., 2016). When microalgae cells are under salt stress, they develop many adaptive strategies as a response to adapt themselves by having different mechanisms such as changes in morphological, physiological and bio-chemical processes (Hiremath & Mathad, 2010). To resist osmotic pressure due to salinity stress, lipid accumulation is favored (Zhu et al., 2016). Moreover, salt stress is the limitation of invasive or competing microorganisms with microalgae, which is very essential especially when the cultivation media is unsterilized wastewater. Therefore, salinity

[2] This chapter is based on the paper: Ermis, H., & Altinbas, M. (2020). Effect of salinity on mixed microalgae grown in anaerobic liquid digestate. Water and Environment Journal.

alteration is a remarkable way to trigger the lipid accumulation which can be converted into biodiesel (Shah et al., 2018). Ishika et al. (2019) investigated the effect of incremental salinity increase on the the biomass productivity of marine (*Tetraselmis suecica*), halotolerant (*Amphora* sp.) and halophilic (*Dunaliella salina*) microalgae; and it was observed that all cultures showed higher lipid productivities (38.4-54.8%) under salinity increase. Moreover, X. Ji et al. (2018) examined that the highest lipid content (32.26%) of *Scenedesmus obliquus* was observed in the presence of 0.20 M NaCl, which was about 2.52 fold higher than when there was no NaCl addition. Moreover,

Anaerobic digestion is a promising technology, which has been practiced for hundreds of years to manage waste and/or to generate energy. During this bacterial breakdown of organic materials, organic nitrogen is converted to ammonia nitrogen, and total phosphorus to orthophosphate by biodegradation of energy rich biomass. Therefore, high concentrations of ammonia and phosphorus are present in the anaerobic digestion effluent in an accessible form for microorganisms (Walker et al., 2009). Even though, direct land application is considered as the most cost-effective solution due to high soil remediation properties in agriculture and reducing the cost of the logistics (Rico et al., 2011), characteristics of the digestion effluent such as high nutrient content, high viscosity, high humidity and high pathogens presence can cause phytotoxic effects in plants (Walker et al., 2009) and/or contaminate the groundwater. Therefore, the application of agricultural land without treatment may constitute a risk where solutions for targeting both reducing adverse effect and increasing product values by valorization should be followed (Ermis & Altinbas, 2019). After liquid and solid phase of digestate is separated, high nutrients in liquid fraction can be recovered/removed by membrane technology, evaporation, and/or stripping where all techniques have high cost and high energy need (Logan & Visvanathan, 2019). On the other hand, coupling the concept of wastewater treatment and microalgae growth is a sustainable solution by providing economical efficiency since microalgae can assimilate nitrogen and phosphate into their biomass as well as inorganic carbon for photosynthesis (J. B. K. Park & Craggs, 2010).

Salinity is a growth-limiting factor that directly affects the biomass productivity of microalgae; however, effects can differ from species to species (Ishika et al., 2017). Solovchenko et al. (2014) observed the effect of salinity on oleaginous microalgae

Nannochloropsis grown in synthetic media and concluded that the culture grown in zero NaCl medium attained the highest biomass which was 0.82 g L^{-1} day^{-1}. Battah et al. (2013) also demonstrated that the growth rate of C. *vulgaris* was remarkably decreased with increased level of salinity, and 27% biomass reduction was observed at 0.45 mM NaCl concentration. T. Wang et al. (2016) were also examined that with the increase of NaCl concentration, growth of C. *protothecoides* was gradually inhibited, particularly stopped growing when NaCl concentration exceeded 0.513 M. Zhila et al. (2011) observed the highest biomass of *B. braunii* at the control culture (no NaCl addition) after 12 days of the experiment where the biomass was 1.1 g L^{-1}. On the other hand, Pandit et al. (2017) observed increased growth rate of *C. vulgaris* with increase dose of NaCl with *C. vulgaris* grown in BG-11 amended with 60 mM NaCl where the highest algal biomass was 0.92 g $L^{-1.}$ Moreover, Salama et al. (2013) observed that *C. mexicana* (0.8 g L^{-1}) and *S. obliquus* (0.65 g L^{-1}) was much higher when BBM was amended with 25 mM NaCl. However, Salama et al. (2013) concluded that the microalgal growth was inhibited by a high concentration of NaCl (100 mM), which subsequently led to a decrease in biomass from 0.8 g L^{-1} to 0.4 g L^{-1}. Moreover, Ji et al. (2018) investigated the salt stress (0.00, 0.01, 0.10, 0.15, 0.20 M) on accumulation of metabolites in *Scenedesmus obliquus* and examined that the biomass and the content of chlorophyll a, b and carotenoids decreased with increasing NaCl concentration. Daneshvar et al. (2018) also examined the effect of salinity on Chlorella vulgaris growth and concluded that low concentration of NaCl (2.125 g L^{-1}) can improve C. vulgaris growth; however, increasing the NaCl concentration from 2.125 up to 17 g L^{-1} resulted in the decrease of biomass and in the presence of 34 g L^{-1} concentration of NaCl, microalgae growth almost stopped.

In this study, mixed algal culture dominated by *Chlorella vulgaris* and *Scenedesmus armatus* were grown in Anaerobic Liquid Digestate (ALD) with the addition of different NaCl concentrations ranging from 0 to 100 mM. Salt stress was applied to observe the growth, biochemical composition, and fatty acid composition of mixed microalgae for being able to combine anaerobic liquid digestate and microalgae cultivation for an effective way to convert high strength dark manure into profitable byproducts as well as to reduce contaminations to environment. To the best of the author's knowledge, there is no other study focusing on the salt stress on mixed algal culture, which reduces the risk of system contamination compared to working with

pure culture and adjust the process easier in difficult conditions by changing its dominance.

3.2 Materials and Methods

3.2.1 Wastewater collection and analysis

The liquid digestate was obtained from full scale plant which was the waste mixture of mechanically/manually separated organic fraction of municipal solid waste (50%), cattle manure (17%), leaching water from solid waste collection vehicles (8%), expired market wastes (4%) and chicken manure (4%). The characterization of ALD was given in previous work by Ermis & Altinbas (2018).The PerkinElmer® Optima ™ 7000 DV ICP optical emission spectrometer was used to analyze the wastewater elements using the standard solutions where calcium (Ca) was 58.1 mg L^{-1}, magnesium (Mg) was 19.1 mg L^{-1}, iron (Fe) was 27.8 mg L^{-1}, manganese (Mn) was 3.9 mg L^{-1}, aluminum (Al) was 9.5 mg L^{-1}, silicon (Si) was 46.7 mg L^{-1}, lead (Pb) was 1.1 mg L^{-1}, boron (B) was 5.8 mg L^{-1}, chromium (Cr) was 5.3 mg L^{-1}, cadmium (Cd) was 0.4 mg L^{-1}, nickel (Ni) was 3.1 mg L^{-1}, silver (Ag) was 4.8 mg L^{-1}, sulfur (S) was 619.7 mg L^{-1}, zinc (Zn) was 0.9 mg L^{-1}, strontium (Sr) was 1 mg L^{-1} and sodium (Na) was 871.4 mg L^{-1}.

3.2.2 Isolation and identification of mixed microalgae

The isolation of mixed culture of microalgae was performed as described in previous work (Ermis & Altinbas, 2018). Isolated wild-type microalgae culture was examined by firstly with light microscopy and mixed culture was morphologically characterized by using microalgae systematics books (Barsanti & Gualtieri, 2014; Proschold & Leliaert, 2007; Bellinger & Sigee, 2015). According to the new generation sequencing results performed by authors (data not shown), it was finalized that the unaxenic mixed culture was dominated by *Chlorella vulgaris* (27%) and *Scenedesmus armatus* (%39).

3.2.3 Experimental design

Effect of salinity on mixed culture were operated in batch culture with triplicate systems using 1000 ml Erlenmeyer flask with 800 working volume with different concentrations of NaCl which were 0 (No NaCl addition, also referred as control), 12 mM, 25mM, 50 mM, and 100 mM. All experiments were started with 2.5 mg chl-a L^{-}

^{1} and 0.5±0.1 g L^{-1} algal biomass. According to the ICP results, 10 % ALD contained 85 mg L^{-1} Na^{+}. Even though, there are many different dissolved salts that contribute to the salinity of water such as chloride, sodium, magnesium, sulfate, calcium, potassium, bicarbonate and bromine, since seawater, a well known salinity standard, have primarily sodium chloride (NaCl) with 33-37 g L^{-1} salinity, salinity was provided by NaCl in this study. Moreover, 0 mM referred to no NaCl addition to 10% ALD as a control cultivation.

The lipid accumulation in microalgae is generally triggered by nitrogen limitation, which mostly occurs during the stationary growth phase of the microalgae (Salim et al., 2013) by limiting the protein synthesis and adjusting different metabolic pathways such as fatty acid synthesis (Msanne et al., 2012). Therefore, mixed culture was harvested at the end of the stationary phase after 18 days by centrifuging at 5000 rpm for 15 minutes. The pellet was washed 3 times to remove the salt and microalgal debris. Analyses were operated to demonstrate and compare the biomass amount, biochemical content and fatty acid compositions with controlled condition.

3.2.4 Analytical methods

Nitrogen was measured as Total Kjeldahl Nitrogen (TKN) and Ammonia (NH_3-N); whereas Phosphorus was measured as Total Phosphorus (TP) and Orthophosphate (PO4). Suspended Solid, TKN, NH_3-N, TP and PO_4-P values analyzed as mg L^{-1} according to Standard Methods (Rice, E.W. Baird, R.B. Eaton, 2017).

Total protein content was estimated by the method of Lowry (Tan et al., 2020) where samples were pre-boiled for 10 minutes with 2N NaOH in 1:1 ratio (v/v) as a pretreatment. 1 ml of cooled sample was taken and 700 µl of Lowry solution was added. After vortexing, samples were put in the dark for 20 minutes at room temperature. Folin solution was prepared 5 minutes before the end of 20 minutes and 100 µl of folin solution was added to the mixture and vortexed. The samples were left in the dark for at least 30 minutes more and read at spectrometer (750 nm) against the distilled water.

Total carbohydrate was determined by Anthrone reagent method (Braga et al., 2018) where 1 ml of the sample was mixed with 2 ml of 75% sulfuric acid and 4 ml of anthrone solution, and incubated for 15 minutes at 100 °C. After samples were cooled down, they were read against distilled water at 630 nm by spectrometer.

Total lipid was calculated by slightly modified version of Bligh & Dyer's method (Saroya et al., 2018). Wet biomass containing 100 ± 5 mg was taken and 1.25 ml of chloroform and 2.5 ml of methanol were added. After 20 minutes of shaking, the samples were vortexed with 1.25 mL of chloroform. The samples were vortexed again by adding 1.25 ml of distilled water. After centrifuging the samples at 3000 rpm for 10 minutes, the lower phase was removed and the samples were evaporated at 70 ° C in a vacuum oven. The glass containing lipid was kept 1 hour at 105 °C to reach constant weight. Triplicate samples were analyzed and the average values were taken.

Pigment content was determined as 2 ml microalgae cells of each strain was centrifuged at 12500 rpm for 5 min. Pellet was taken and suspended with 2 ml methanol (90%). The mixture was incubated in water bath at 80^{o}C for 5 min. The steps were continued by centrifugation at 12500 rpm for 5 min. The supernatant was transferred and measured by spectrophotometer UVvis at 470 nm, 665 nm and 655 nm against the solvent (methanol) blank. The concentration of chlorophyll a, chlorophyll b and total carotenoids were calculated using the equations below (Sumanta et al., 2014):

$$\text{Chlorophyll a } (Ch_a) = 16.72(A_{665}) - 9.16(A_{655}) \quad (3.1)$$

$$\text{Chlorophyll b } (Ch_b) = 34.09(A_{655}) - 15.28(A_{665}) \quad (3.2)$$

$$\text{Total carotenoid content} = [1000(A_{470}) - 1.63Ch_a - 104.96Ch_b]/221 \quad (3.3)$$

For Fatty Acid Methyl Ester (FAME) analyse, Laurens et al. (2014), and El-Shimi et al. (2013) procedures were followed for acid catalyzed in-situ transesterification. 5-10 mg of microalgal biomass were put into the vials. Meanwhile, methyl tridecanoate (C13: 0ME) internal standard were prepared and the heat block was preheated to 85 ° C. Afterwards, 20 µL L of C13:0 ME (10 mg / mL), 200 µL L chloroform: methanol (2: 1, v / v), and 300 µL 0.6M HCl:methanol (methanolic hydrochloric acid) were added in to vials with 5- 10 mg dry algal samples and closed with crimple and vortexed. The samples were placed in the direct heat block without waiting at room temperature after vortex. They were incubated for 1 hour at preheated 85 °C heat block. End of the incubation, 1 ml of hexane was added via a gas-tight syringe in to the samples and samples were kept at room temperature for 1-4 hours to observe phase separation. The upper phase was measured by Gas Chromotography (GC). Shimadzu AOC-20i, GC 2010 model Gas Crotomography with CN100 capillary column (Teknokroma, Barcelona, Spain) with a length of 100 m × 0.25 mm and an internal diameter of 0.2

μm film thickness were used. The carrier gas was helium and the hydrogen gas flow was 40 ml / min whereas air gas flow was 400 ml min^{-1}. FAME Reference Standard (Catalog No: FAMQ-005) used in this study was with 37 components.

3.2.5 Mass Balance Calculations

Mass balance was performed to confirm the correlation between nutrient removal and biomass yield as described in previous work (Ermis & Altinbas, 2018). Stoichiometric equation below was used to calculate the theoretical microalgae formation (Ebeling et al., 2006):

$$16\,NH_4^+ + 92\,CO_2 + 92\,H_2O + 14\,HCO_3^- + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 106\,O_2 \quad (3.4)$$

3.3 Results and Discussion

3.3.1 Impact of NaCl concentration on cell growth and biomass productivity

The results clearly indicated that the algal biomass amount gradually decreased from 1.45 g L-1 to 0.9 g L-1 with increased salinity from 0 mM to 100 mM NaCl (Table 3.1). Due to the highest biomass growth observed in control operation with 1.45 g L-1, it was attributed that ALD had the enough salt concentration that supported growth and metabolic activities of microalgae. In this study, lowest growth was also observed at 100 mM NaCl concentration. It should be noted that all studies mentioned above were conducted within synthetic media and with pure microalgal cultures; therefore, this study demonstrated a cost-effective approach towards the cultivation of mixed microalgae grown in wastewater with high biomass yield. Mixed microalgal culture could be able to perform better than pure culture by changing species dominance and/or biochemical compositions due to stress conditions caused by not only dark, high ammonicial and particulate rich digestate; but other stress conditions such as salinity.

Table 3.1 : Impact of salinity on cell growth and nutrient removal efficiencies.

NaCl Concentration (mM)	Algal biomass (g L^{-1})	PO_4-P removal (%)	NH_3-N removal (%)
(Mean±SD*), n=2			
Control (0)	1.45	80	92
12	1.32	78	85
25	1.26	75	83
50	1.18	71	76
100	0.9	67	72

*SD: Standard Deviation, p>0.05

In this study, the highest nutrient removal for NH3-N and PO4-P were observed when there were no NaCl addition (0 mM) which were 92% and 80%, respectively; and gradually decreased with increased NaCl concentrations. The decrease of nutrient removal with increased salinity could be due to the photosynthetic rate inhibition by high C/L ions (Fisarakis et al., 2001), which eventually reduce nitrogen uptake by leading to decrease of the algal growth.

3.3.2 Mass Balance and free ammonia (NH_3) inhibition

The original pH of ALD was 9 and pH increased to 10.5 at the end of 18 days' cultivation as the dissolved CO_2 was removed from the ALD through the photosynthesis. Therefore, ammonia volatilisation should be considered under these circumstances. Under stable conditions, NH_3 (ammonia) and NH_4 (ammonium) will be both present in wastewater at an equilibrium point that is depended mostly on pH and temperature. The original pH of ALD in this study was 9 which was already at an equilibrium point. However, algal growth with photosynthesis increases the pH; hence, the amount of free ammonia is expected to increase. It was assumed that there was no nitrification since nitrifying bacteria are very sensitive microorganisms where *Nitrosomonas* and *Nitrobacter* has an optimal pH between approximately 7.0 and 8.0 (EPA, 2002) which was also supported by Skadsen (2002) who reported that a greater pH than 9 can be used to reduce the occurrence of nitrification. Moreover, in this study, all ammonium was consumed by microalgae not by bacteria according to the no presence of bacteria was observed microscopically due to high pH in all batch operations.

Ammonia toxicity explained as a deterioration of the thylakoid transmembrane proton gradient where NH_3 can freely diffuse across cell membranes (Azov & Goldman, 1982). However, the NH_3 inhibition on algal growth is still unclear and not well documented for many algal cultures. Gutierrez et al. (2016) observed free ammonia inhibition on two of the four microalgae species which were *N. oleoabundans* and *D. tertiolecta.* Both species showed 50% growth inhibition occurring at 8 mg L^{-1} free ammonia, even tough for both species inhibitions started to occur at 2.3-3.3 mg L^{-1} NH_3. However, the two remaining species in their study, *C. sorokiniana* and *N. oculata,* did not show growth inhibition due to NH_3 which indicates that NH_3 inhibition can be dependent on the algal culture. Meanwhile, Azov & Goldman (1982) reported that 20.4 mg L^{-1} free ammonia led to 50% reduction in photoassimilation *of Scenedesmus obliquus, Phaeodactylum tricornutum* and *Dunaliella tertiolecta.* Moreover, Källqvist & Svenson (2003) examined that the specific toxicity of free ammonia was 32.8 mg ammonia nitrogen/L to microalga *Nephroselmis pyriformis*. Lin et al. (2007) isolated two microalgae species, *Chlorella pyrenoidosa* and *Chlamydomonas snowiae,* having a high ammonia leachate pond and the results indicated that the algal growth was inhibited by high leachate concentrations which appears linked to high ammonia (670 mg L^{-1}) but there was no proof related to free ammonia inhibition.

In this study, mass balance calculations were applied to confirm the correlation between nutrient removal and biomass yield. The theoretical NH_4-N removal was calculated according to the experimental observed biomass formed where biomass was expressed as volatile suspended solid (VSS). The experimental observed biomasses were 942, 795, 623, 567 and 520 mg L^{-1} when the salinity were 0, 12, 25, 50 and 100 mM NaCl, respectively. As it can be seen in Table 3.2, there was an unknown free ammonia observation which was 26, 28, 35, 34, and 33 mg NH_3 L^{-1} for 0, 12, 25, 50 and 100 mM NaCl, respectively.

This free ammonia amount for all batch operations were similar to the inhibition amount observed by Azov & Goldman (1982) and Källqvist & Svenson (2003) which might indicate that in this study there might be a possible ammonia inhibition on algal culture. However, since the amount of free ammonia for all batch operations were almost congruent due to the similar final pH (26-35 NH_3 L^{-1}); the amount of biomass decreasing with increasing salinity could be interpreted as a salt inhibition where the

algal biomass decreased from 1.45 g L^{-1} to 0.9 g L^{-1} with increased salinity from 0 mM to 100 mM NaCl.

Table 3.2 : Theoretical and experimental results for different salinity concentrations.

NaCl Concentration (mM)	0	12	25	50	100
	(Mean±SD*), n=2				
Experimental observed biomass (mg SS L^{-1})	1450	1320	1260	1180	900
Experimental observed biomass (mg VSS L^{-1})	942	795	623	567	420
Experimental observed fixed solids (mg VSS L^{-1})	508	525	637	613	480
Influent NH_4-N (mg L^{-1})	95	93	89	95	91
Effluent NH_4-N (mg L^{-1})	10	15	15	26	26
NH_4-N removal (mg L^{-1})**	85	78	74	69	65
Theoretical NH_4-N removal according to experimental observed biomass (mg NH_4-N L^{-1})	59	50	39	35	32
Unknown free ammonia (mg NH_3 L^{-1})	26	28	35	34	33
Initial pH	9.1	9.4	9.4	9.5	9.6
Final pH	10.9	10.5	10.7	10.3	10.1

* SD: Standard Deviation, p>0.05
**Subtraction of influent NH_4-N from effluent NH_4-N

3.3.3 Impact of salinity on biochemical composition

Extreme high salinity introduced to microalgae can inhibit the cell growth and change the shape and structure of microalgal cells, due to the water pressure between media and cells (Zhu et al., 2016). However, this increased concentration of NaCl increases the lipid amount while decreasing the biomass amount. In this study, the increase of NaCl concentration in 10% ALD from 0 (no addition) to 50 mM enhanced the total lipids amount from 13.5% to 38.4% and the carbohydrates amount from 26.1% to 36.2%. (Figure 3.1).

Church et al. (2017) also observed increased total lipid content from 11.5% to 16.1% while also increased saturated portions of fatty acids in *C. vulgaris* grown in artificial wastewater with salinity stress. Salama et al. (2013) observed highest lipid content of *C. mexicana* and S. obliquus as 37% and 34 % respectively when BBM was amended with 25 mM NaCl. There are more other studies also observed increase of lipid while decrease of algal biomass with an excess of higher salinity (K. SUJATHA AND P. NAGARAJAN, 2014; Rao et al., 2007; Ruangsomboon, 2012; Zhila et al., 2011). The imbalance of salts such as Na^+ lead to an osmotic stress and formation of Reactive Oxygen Species (ROS) which are vital for the algal cellular defense mechanism. Therefore, the occurrence of ROS causes reduced growth due to photo-inhibition

where an increase rate of lipid synthesis occurs under similar stress conditions where lipids play an important role in destructing of the accumulated ROS (Srivastava et al., 2017).

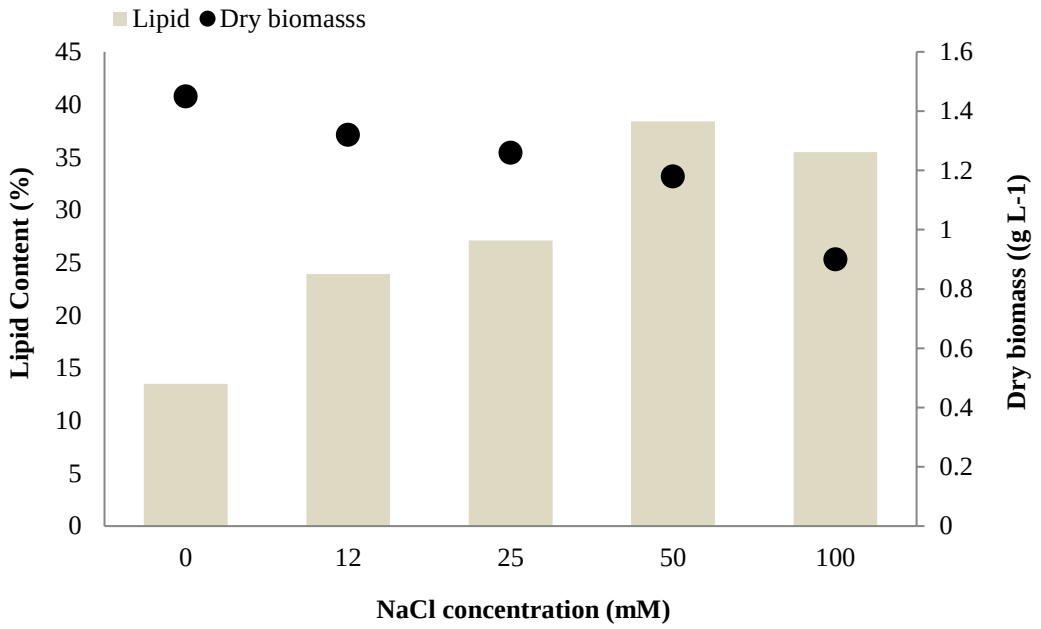

Figure 3.1 : Effect of NaCl on lipid content (%) and dry biomass (g L^{-1}) ($p>0.05$).

Gill et al. (2002) mentioned that soluble sugars contribute an important role in controlling the osmotic regulation of cell. Therefore, the increase in the carbohydrate amount helps the microalgae to survive under saline conditions. Torabi (2014) also mentioned that carbohydrates accumulate under salt stress and play a major role in osmo-protection, osmotic adjustment, carbon storage, and radical scavenging. In this study, when salinity increased to 50 mM NaCl, both lipid and carbohydrate amount increased to 38.4% and 36.2%, respectively (Figure 3.2).

The reason of synchronic increase in both carbohydrates and lipids amount is that their pathways of synthesis are parallel where the energy is first stored in the form of carbohydrates and then the excess is converted into lipids (BenMoussa-Dahmen et al., 2016). However, in this study more than 50 mM NaCl did not improve neither the amount of lipid nor carbohydrates which could be threshold of salinity for the mixed culture.

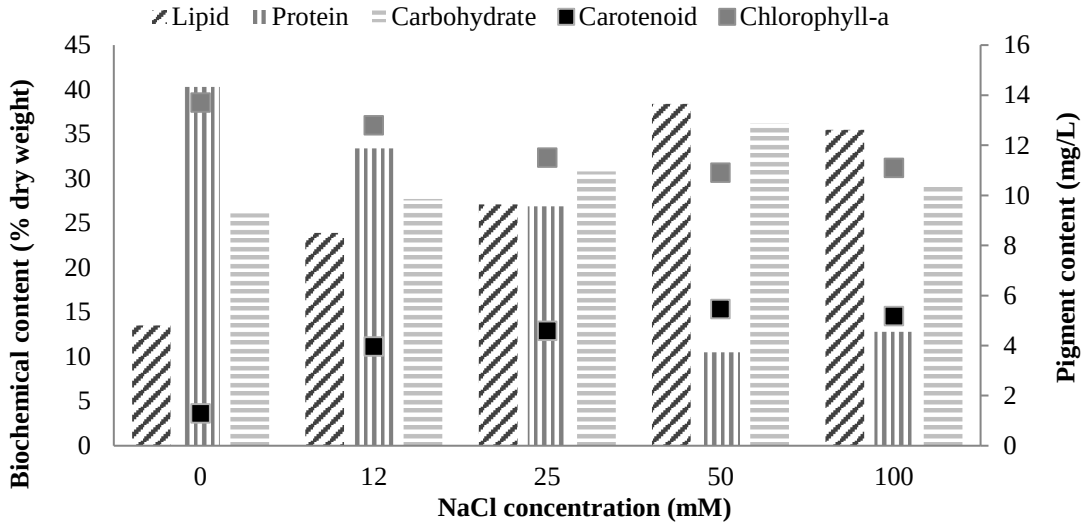

Figure 3.2 : Effect of NaCl on biochemical composition and pigment amount ($p>0.05$).

On the other hand, the proteins amount decreased from 40.3% to 10.5% up to 50 mM NaCl then increased to 12.8 % at 100 mM NaCl. This parallel increase with lipid and carbohdyrate and simultaneous decrease with protein was also observed in the literature (Hiremath & Mathad, 2010; Kirrolia et al., 2011). Dittami et al. (2011) explained this protein decrease that during salinity stress, down regulation of genes involved in protein synthesis occurs which activates gene related to protein degradation in algae. Also, this decrease might be due to the increasing activity of acid and alkaline proteases in order to keep osmotic stress during NaCl stress (Plant & Centre, 2002). Not only the protein content, but also the chlorophyll-a content was decreased from 18.5 mg L^{-1} to 7.7 mg L^{-1} with increased salinity. Chlorophyll is the primary target to salt toxicity limiting net assimilation rate, causing decreased photosynthesis along with low biomass production (Pandit et al., 2017). Moradi and Ismail (2007) also determined that salinity stress cause decrease in the rate of photosynthesis and hence, lowers chlorophyll and protein content. Moreover, within the stress conditions, a small fraction of the absorbed light energy only enters into the photosystem pathways and subsequently decreases the total quantum yield (Srivastava et al., 2017). Therefore, the chlorophyll-a content in mixed culture was highest at control operation (without NaCl addition) with 18.5 mg L^{-1}. Reduction of chlorophyll content indicated that there was a photo-protection mechanism through reducing light absorbance by decreasing chlorophyll contents (Taïbi et al., 2016). According to Saha et al. (2013) the increase of (Car/Chl-a) ratio is a very good stress response, which

reduce the antenna size that may be a protective function against photo-oxidative damage (Pancha et al., 2015). In this study, the Car/Chl-a increased until 50 mM NaCl, and started to decrease at 100 mM NaCl. In contrast to chl-a content, total carotenoid content was increased 66% with 50 mM NaCl (Figure 3.2). K. Sujatha and P. Nagarajan (2014) examined that when salt stress was applied to *Spirulina platensis*, chlorophyll and protein amount were decreased, whereas carotenoids and lipid amount were increased due to inhibition of photosynthetic and respiratory systems. Cifuentes et al. (1996) mentioned that the relationship among salinity, growth and carotenogenesis is complex and added that there is a cross connection between growth and the accumulation of carotenoids. The decrease of carotenoid contents in this study indicated that the protection by carotenoid was one of the important mechanisms under salt stress.

3.3.4 Effect of salinity on fatty acids profile

After biochemical analyses of mixed culture, the fatty acid compositions were determined by in-situ transesterification. Microalgal lipids are composed of saturated, monounsaturated, and polyunsaturated fatty acids. The results revealed that the productivity of lipid from microalgae was dependent upon the concentration of NaCl in culture media. Salinity level at 50 mM NaCl was found to increase overall lipid content through increase of SFA and PUFA in mixed algal culture where SFA increased from 44.2% to 53%, and MUFA increased from 13.5% to 18.6% (Figure 3.3).

Zhila et al. (2011) mentioned that considerable variations were found in the content of saturated fatty acids in their studies where at at 0.7 M NaCl, their total percentage increased to 14.1 .

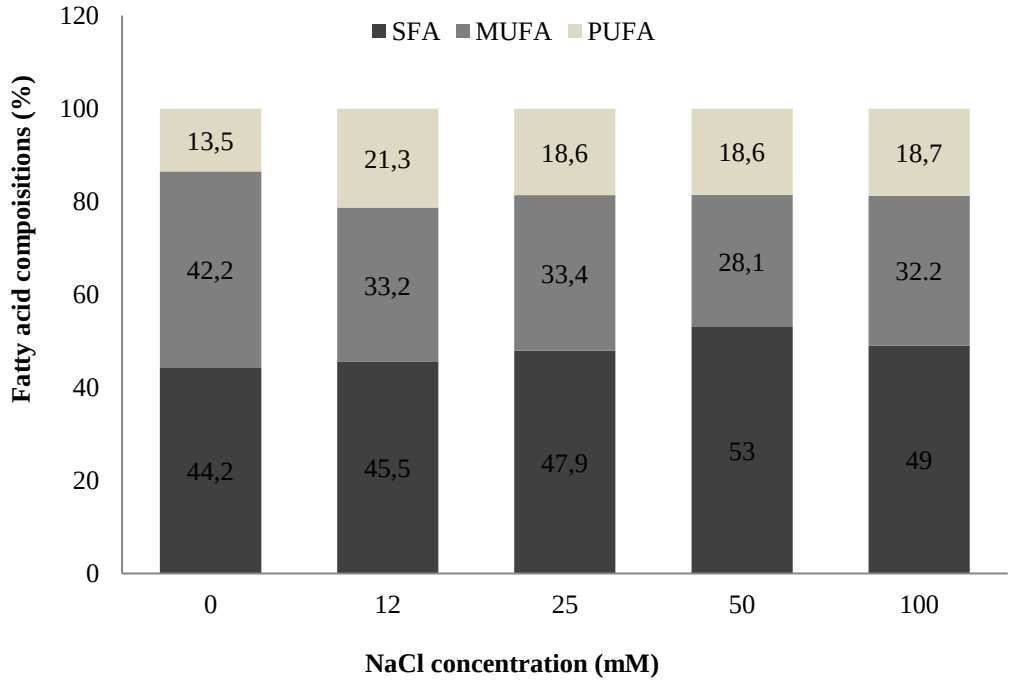

Figure 3.3: Effect of NaCl on fatty acid composition ($p>0.05$).

In this study, salinity significantly enhanced the synthesis of neutral lipids specifically triacylglycerides (TAGs) in the form of secondary metabolite which was also observed by Srivastava & Goud (2017). The fatty acids profile showed an increase of total saturated fatty acids (SFA) with the new appearance of traces of C20:0, C21:0 and C22:0, which indicates that the salinity can affect the composition of the cell membrane (Table 3.3). The SFU content reached maximum (53%) at 50 mM NaCl and started to decreased at 100 mM NaCl (49%). Meanwhile, there was a decrease of mono-unsaturated fatty acid (MUFA) an increase of poly-unsaturated fatty acids (PUFA).

High percentages of linolenic acid (C18:2) are usually observed in algal biodiesel (Koutra et al., 2018) because, compared with most vegetable oils, algal oil extracted from freshly harvested biomass is exceptionally rich in PUFAs, since PUFAs in microalgae enables them to remain metabolically active and survive the stress conditions that often occur in their habitats (Sobczuk & Chisti, 2010). Campenni' et al. (2013) observed that when *Chlorella* sp. exposed to salinity stress, fatty acids profiles were lower than this study where C16:0 was 23.30%, C18:0 was 1.71% and C18:3 was 11.12%. Similar FAME profile on *Chlorella* sp. was also reported by L. Wang et al. (2010), Li et al. (2011), Lang et al. (2011) and Shekh et al. (2016).

Table 3.3 : FAME profiles for mixed microalgae under different salt stress.

Fatty Acids	NaCl Concentration (mM) (Mean±SD*), n=2				
	0	12	25	50	100
	Fatty Acid Composition (%, w/w)				
Saturated fatty acids (SFU)					
14:0	3.5	3.1	0.5	6.7	8.3
15:0	1.5	1.8	1.3	1.8	1.1
16:0	32.4	23.82	28.9	31.4	25.9
18:0	4.7	10.5	9.8	9.1	6.6
20:0	-	-	1.3	-	1.2
21:0	-	-	1.8	0.3	1.9
22:0	0.6	1.4	1.0	0.9	0.9
23:0	-	1.0	0.7	0.6	0.8
24:0	0.9	3.9	2.5	2.2	2.2
Total SFU	**43.6**	**45.5**	**47.9**	**53**	**49**
Monounsaturated fatty acids (MUFA)					
14:1	12.8	2.2	3.3	1.7	4.6
15:1	6.6	11.1	8.4	8.4	6.9
16:1	5.7	-	2.3	2.1	2.1
18:1n-9t	10.1	9.6	10.5	8.6	10.7
18:1n-9c	7.6	10.3	8.9	7.6	7.8
Total MUFA	**42.9**	**33.2**	**33.4**	**28.4**	**32.2**
Polyunsaturated fatty acids (PUFA)					
18:2 9t	4.13	3.23	3.19	3.52	2.71
18:2 9c	6.82	10.87	9.70	10.33	10.83
18:3	1.34	2.05	1.46	1.98	1.40
18:3n-c	0.99	-	0.71	0.77	0.80
20:2	-	-	0.90	0.51	1.55
20:3	0.26	5.17	2.65	1.45	1.46
Total PUFA	**13.55**	**21.32**	**18.62**	**18.57**	**18.75**

*SD: Standard Deviation, p>0.05

In this study, linoleic acid (C18:2) increased up to 10.9% by the addition of 12 mM NaCl while linolenic acid (C18:3) did not change drastically and ranged between 1.3-2%. Maximum limit of polyunsaturated fatty acid content for C18:3 was determined as < 12% (w/w) by European biodiesel standard EN14214 because the unsaturation grade affects the cold flow, stability and ignition quality of diesel fuel (European Committee for Standardization, 2003). Therefore, for this study, it can be concluded that under salinity stress, mixed culture dominated by C.*vulgaris* and S. *armatus* can be considered suitable producers of saturated fatty acids which is easily convertible to biodiesel.

3.4 Conclusion

This study demonstrated that 50 mM NaCl salt concentration was an effective stress that could increase not only the lipid content but also improve the fatty acid composition by increasing its SFU content. Salt stress is the limitation of invasive or competing microorganisms with microalgae which is crucial when the cultivation media is unsterilized wastewater as in real scale studies. Therefore, this study provided the cost-effective strategy towards the cultivation of mixed microalgae consortia, which could be further converted in to biofuels along with the digestate treatment. It was observed that mixed microalgal culture could be able to perform better than pure culture by changing species dominance and/or biochemical compositions due to stress conditions caused by not only dark, high ammonicial and particulate rich digestate; but also the salinity; therefore, reduced the risk of system contamination and increase the process feasibility. Microalgae is one of the promising candidate to recover nutrients from anaerobic liquid digestate by assimilating nitrogen and phosphate into their biomass (Franchino et al., 2013) and help to bring anaerobic liquid digestate into the economy. This study might help to be a solution for targeting both reducing adverse effect of anaerobic liquid digestate by mixed microalgae and increasing product values by valorization and give an idea that digestate can be diluted with sea water instead of tap water in the future studies.

4. EFFECT OF IRON AND MAGNESIUM ADDITION ON POPULATION DYNAMICS AND HIGH VALUE PRODUCT OF MICROALGAE GROWN IN ANAEROBIC LIQUID DIGESTATE[3]

4.1 Introduction

Microalgae, having no need for arable land, is an attractive source of high value products by their rapid growth. However, the main obstacle to the commercialization of algae-derived products is the high cost of production (Borowitzka, 2013). To overcome this high capital investments and operation costs, high-value co-products such as pigments, proteins, lipids, and carbohydrates should be produced to improve the economics of microalgae applications (Chew et al., 2017) along with wastewater treatment, which is a source to obtain nutrients at a low cost. Anaerobic digestion is biodegradation of nutrient rich biomass, which is commonly used for organic matter stabilization and biogas production. Unfortunately, this process leads to produce Anaerobic Liquid Digestate (ALD) (Zuliani et al., 2016), which is extremely high in ammonia and orthophosphate. Even though direct land application is considered as the most cost-effective solution due to high soil remediation properties in agriculture and reducing the cost of the logistics (Rico et al., 2011), characteristics of the digestion effluent can cause phytotoxic effects in plants and/or contaminate the groundwater (Ermis & Altinbas, 2019). In this aspect, microalgae can be efficiently grown in liquid digestate and stabilize the effluent without any further treatment.

The effect of macro elements such as nitrogen and phosphorus on microalgae and its biochemical composition has been the focus of research. However, other macro elements such as iron and magnesium play also a critical role in a variety of metabolic pathways important for microalgae. For instance, iron (Fe) is a crucial micronutrient for almost all living organisms because of its role in metabolic processes such as DNA synthesis, respiration, and photosynthesis. It works as a cofactor for enzymes due to

[3] This chapter is based on the paper: Ermis, H., Guven-Gulhan, U., Cakir, T., & Altinbas, M. (2020). Author Correction: Effect of iron and magnesium addition on population dynamics and high value product of microalgae grown in anaerobic liquid digestate. Scientific Reports, 10(1), 1-2.

its ability to gain and lose electrons (Rout & Sahoo, 2015). Magnesium (Mg), on the other hand, occupies a strategic position as the central element of the chlorophyll molecule, and all microalgal species have an absolute need for this element (Farhat et al., 2016). Although, the deficiency of iron and magnesium on microalgal growth and photosynthetic efficiency has been investigated, only a few studies focus on the influence of iron and magnesium supply on the biochemical composition. They were all in synthetic media, and only for a limited number of species.

Tap water is mostly used for diluting the wastewater, and certain type of bacteria appeared in tap water had been examined for their effects on microalgae growth (Lian et al., 2018). It is shown that short-term changes in growth conditions can reduce the number of those undesired microorganisms, which is important for non-axenic cultures grown in unsterilized wastewater. Undefined mixed algal culture isolated from nature is a black box that is needed to be enlightened since each undefined culture is unique and specific to its environment. Non-axenic mix microalgae consortia can perform better than unicellular culture with regard to dominancy change due to stress conditions, which prevents the culture loss and lowers the risk of system contamination. However, the mixed consortia should be observed cautiously to assure that microalgae concentration in the consortia remains higher than the bacteria culture to prevent the disappearance of algal cells (Stiles et al., 2018).

Large-scale taxonomic identification has been a challenge for mixed culture composition analysis. Metabarcoding is a novel terminology (Taberlet et al., 2012) that has been used for the large-scale taxonomic identification of complex environmental samples (Marcelino & Verbruggen, 2016; Sauvage et al., 2016; Smith et al., 2017). DNA metabarcoding has been argued to be the next generation tool for detecting mixed species biodiversity in ecological studies and aquatic ecosystems (Evans et al., 2016; Valentini et al., 2016). Multi-marker metabarcoding, the use of multiple marker regions, is preferred to characterize mixed cultures that include prokaryotes and eukaryotes (Faluaburu et al., 2019; Marcelino & Verbruggen, 2016). A wide range of prokaryotic and eukaryotic organisms can be identified via 16S rRNA, 18S rRNA and 23S rRNA barcoding analysis. More specific markers are also available such as tufA region, which was found to be an effective marker for the identification of prokaryotic (the cyanobacteria) and eukaryotic algae (Sauvage et al., 2016).

The aim of this study was to elucidate how population dynamics changed, and which species were favored by Fe and Mg supplementation. The effect of these elements on biochemical composition of mixed culture and nutrient removal efficiency was also investigated. This study is the first attempt to analyze undefined algal microbiome grown in anaerobic digestate, and it will help to understand how the dynamics of symbiotic life changes if macro elements are added to the ALD. A multi-marker metabarcoding approach was used for the characterization of microorganisms in the mixed cultures in anaerobic liquid digestate by analyzing 16S rRNA, 18S rRNA, 23S chloroplast RNA and tufA marker regions. To the best of the authors knowledge, there is no other study that uses multi-marker metabarcoding approach and reports confirmation of results with 4 markers simultaneously for undefined mixed culture of anaerobic digestion, which is important for revealing the molecular diversity in detail. The results of this study will contribute to the efforts to combine digestate treatment with microalgae cultivation for an effective conversion of high strength dark wastewater into high value byproducts.

4.2 Materials and Methods

4.2.1 Wastewater collection and analysis

The liquid digestate was obtained from a full scale plant decomposing the waste mixture of mechanically/manually separated organic fraction of municipal solid waste (50%), cattle manure (17%), leaching water from solid waste collection vehicles (8%), expired market wastes (4%) and chicken manure (4%). The characterization of ALD was given in a previous work by Ermis and Altinbas (2018). The PerkinElmer Optima 7000 DV ICP (Inductively Coupled Plasma) optical emission spectrometer was used to analyze the wastewater elements using the standard solutions where calcium (Ca) was 58.1 mg L^{-1} , magnesium (Mg) was 19.1 mg L^{-1}, iron (Fe) was 27.8 mg L^{-1}, manganese (Mn) was 3.9 mg L^{-1}, aluminum (Al) was 9.5 mg L^{-1}, silicon (Si) was 46.7 mg L^{-1}, lead (Pb) was 1.1 mg L^{-1}, boron (B) was 5.8 mg L^{-1}, chromium (Cr) was 5.3 mg L^{-1} , cadmium (Cd) was 0.4 mg L^{-1}, nickel (Ni) was 3.1 mg L^{-1}, silver (Ag) was 4.8 mg L^{-1}, sulfur (S) was 619.7 mg L^{-1}, zinc (Zn) was 0.9 mg L^{-1}, Sr was 1 mg L^{-1} and sodium (Na) was 871.4 mg L^{-1}.

4.2.2 Isolation and identification of mixed microalgae

The isolation of mixed culture of microalgae was performed as described in a previous study (Ermis and Altinbas, 2018). Algal cultures were firstly inoculated in M_8 media containing the following components (per liter): KNO_3 (3000 mg L^{-1}), KH_2PO_4 (740 mg L^{-1}), $Na_2HPO_4.2H_20$ (260 mg L^{-1}), $CaCl_2.2H_2O$ (13 mg L^{-1}), Fe EDTA (10 mg L^{-1}), $FeSO_4.7H_2O$ (130 mg L^{-1}), $MgSO_4.7H_2O$ (400 mg L^{-1}), and 1 mL Micronutrients consisting of $Al_2(SO_4)_3.18H_2O$ (3.58 g L^{-1}), $MnCl_2.4H_20$ (12.98 g L^{-1}), $CuSO_4.5H_2O$ (1.83 g L^{-1}), and $ZnSO_4.7H_2O$ (3.2 g L^{-1}) (Lian et al., 2018). Afterwards, mixed culture was inoculated in diluted ALD (2%) for acclimation. The culture was inoculated into the same diluted wastewater repeatedly and monitored by microscopic observations frequently. Before the beginning of the each batch cultivation, it was assured that the culture was healthy.

Isolated wild-type microalgae culture was firstly checked by light microscopy, and mixed culture was morphologically characterized by using microalgae systematics books. Afterwards, next generation sequencing was performed.

4.2.3 PCR amplification and sequence analyses of 16S rRNA, 18S rRNA, 23S rRNA and tufA

Molecular confirmation of isolates was performed via next generation sequencing of 16S/18S/23S rRNA and tufA marker regions. Genomic DNA from different mixed microalgae culture samples was isolated and high-throughput sequencing analysis was applied to each sample. Targeted amplicon libraries were constructed with universal V4 region primers [515f (F), 5'-GTGCCAGCMGCCGCGGTAA-3' and 806r (R), 5'-GGACTACHVHHHTWTCTAAT-3' for 16S, TAReuk454FWD1 (F), 5'-CCAGCASCYGCGGTAATTC-3' and TAReukREV3 (R), 5'-ACTTTCGTTCTTGATYRA-3' primers for 18S rDNA, p23SrV_f1 (F), 5'-GGACAGAAAGACCCTATGAA-3' and p23SrV_r1 (R), 5'-TCAGCCTGT-TATCCCTAGAG-3' primers for 23S rDNA, (F) 5'-TGAAACAGAAMAWCGTCATT-3' and (R) 5'-CCTTCNCGAATMGCRAAW-3' primers for elongation factor tufA. Purified-amplicon libraries were sequenced using an Illumina MiSeq platform (2x300 paired-end reads).

4.2.4 Bioinformatic analysis

Sequence data from marker regions were analysed with Quantitative Insights Into Microbial Ecology 2 program (QIIME2 ver. 2019.4 (Bolyen et al., 2019)). After demultiplexing raw reads with cutadapt plug-in, denoising and generation of amplicon sequence variants (ASVs) were performed using the Divisive Amplicon Denoising Algorithm (DADA2). Denoising step includes chimera detection and removal, sequence error elimination, singleton exclusion and sequence trimming based on sequence quality graph and expected amplicon size. The resulting sequences were then classified with the SILVA reference database (Quast et al., 2013; Yilmaz et al., 2014)(132_release of Dec 13, 2017) and tufA database (Sauvage et al., 2016). For 16S rDNA analysis, SILVA database trimmed to the V4 region (515F/806R) was used for taxonomic classification. Taxonomic bar plots are given in Figure A1.

After taxonomic classification, alpha and beta diversity analysis among samples were performed via "qiime diversity core-metrics-phylogenetic" function. To allow for a comparison between the analysis of different samples, we used a user-specified sampling depth per sample per marker analysis. The sample sequences were rarefied (sub-sampled) to 45000, 48000, 50000 and 7000 reads for 16S, 18S, 23S and tufA analyses respectively (Table A1).

Microalgae consortia in this study was identified via amplicon sequencing of small subunit of eukaryotic nuclear ribosomal DNA (18S rDNA), small subunit of prokaryotic ribosomal DNA and eukaryotic chloroplast DNA (16S rDNA), large subunit of eukaryotic chloroplast DNA (23S rDNA), and elongation factor EF-Ttu (tufA) gene of prokaryotic (cyanobacteria) and eukaryotic algae.

Alpha diversity of microbes, including phylogenetic diversity was tested to document whether the internal diversity differs among different environmental stress conditions, including iron and magnesium stress. For this purpose, several alpha diversity parameters were tested. Alpha diversity indices (observed ASV richness, Shannon diversity, Faith's phylogenetic diversity, and Pielou's evenness) of rarefied samples were calculated in QIIME2 with q2-diversity plug-in. Next, maximum-likelihood phylogenetic trees were constructed with "align-to-tree-mafft-fasttree" function of phylogeny plug-in, which uses FastTree2 Next, maximum-likelihood phylogenetic trees were constructed upon masked MAFFT alignment of representative sequences, and rooted phylogenies were inferred via "fasttree" and "midpoint-root" functions of

phylogeny plug-in, which uses FastTree2 (Price et al., 2010). Phylogenetic trees, constructed using sequences with most abundant ASVs (minimum total feature frequency of 100), were uploaded to the Interactive Tree of Life (iTOL) tool (Letunic & Bork, 2016)for the illustration of the taxonomic community compositions (Figure A2).

Beta diversity among the three samples was analyzed as well in order to test statistically whether microbial composition differed among the three conditions. Beta diversity metrics were calculated in QIIME 2 with q2-diversity plug-in and beta function. Principal coordinates analysis (PCoA) was employed based on Bray-curtis distance matrix in order to detect the variation in the microbial communities of samples (Figure A3).

Principal component analysis (PCA) was also performed in MATLAB R2015a. The PCA plot was constructed in order to demonstrate the differential effects of culturing conditions on the mostly detected microbial species identified in the mixed culture. The percent compositions of lipid, protein, carbohydrate and pigments (carotenoid and chlorophyll) under magnesium and iron added cultures, along with the percent relative abundances of detected microbial species were standardized by z-score normalization prior to PCA analysis.

4.2.5 Experimental design

The iron and magnesium source and the starting point concentrations were determined based on the synthetic media study by authors (data not shown). Ferrous sulfate ($FeSO_4.7H_2O$, Molar Mass: 278 g/mol) starting amount was based on the amount available in M_8 media (130 mg L^{-1}) and gradually increased to 1000 mg L^{-1} of $FeSO_4$; and magnesium sulfate ($MgSO_4.7H_2O$, Molar Mass: 246.5 g/mol) starting amount was based on the amount contained in BG-11media (130 mg L^{-1}) and gradually increased to 1000 mg L^{-1} of $MgSO_4$ (Crofcheck et al., 2012). The mixed algal culture was operated in batch culture with triplicate using 1000 ml Erlenmeyer flask with 800 ml working volume with different doses of $FeSO_4$and $MgSO_4$ concentrations. The molarity calculations showed that the $FeSO_4$ concentrations were 0.6, 1.8, 3.1, and 4.5 mM (130, 400, 700, and 1000 mg L^{-1} $FeSO_4$); and the $MgSO_4$ concentrations were 0.4, 2, 3.5 and 5.1mM (75, 400, 700 and 1000 mg L^{-1} $MgSO_4$). The best growth dilution ratio was selected as 10% for mixed culture in a previous study by authors (data not shown) and according to the ICP results, the iron amount was negligible for 10% ALD;

hence, 10% ALD was assumed as control batch (0 mM concentration) for both iron and magnesium batch.

All experiments were started with 2.5 mg chl-a L^{-1} and 0.5±0.1 g L^{-1} algal biomass and harvested at the end of the stationary phase after 16 days by centrifuging at 5000 rpm for 10 minutes. Cultures were kept in an acclimation cabinet under approximately 150 µmol photon m^{-2} s^{-1} continuous illumination measured with a light meter (Hansatech QRT1 Quantitherm), at 25 °C ± 2 °C during the acclimation period and during the experiments, where continuous light was provided to increase algal growth. All batches were monitored for more days to observe the death phase to confirm that the stationary phase ended.

4.2.6 Analytical methods

Nitrogen was measured as Total Kjeldahl Nitrogen (TKN) and Ammonia (NH_3-N); whereas Phosphorus was measured as Total Phosphorus (TP) and Orthophosphate (PO4). Suspended Solid, TKN, NH_3-N, TP and PO_4-P values were analyzed as mg L^{-1} according to Standard Methods (Rice, E.W. Baird, R.B. Eaton, 2017).

Total protein content was estimated by the method of Lowry [52] where samples were pre-boiled for 10 minutes with 2N NaOH in 1:1 ratio (v/v) as a pretreatment. 1 ml of cooled sample was taken and 700 µl of Lowry solution was added. After vortexing, samples were kept in the dark for 20 minutes at room temperature. Folin solution was prepared 5 minutes before the end of 20 minutes, and 100 µl of folin solution was added to the mixture and vortexed. The samples were left in the dark for at least 30 minutes more and read at spectrometer (750 nm) against the distilled water.

Total carbohydrate was determined by Anthrone reagent method [53] where 1 ml of the sample was mixed with 2 ml of 75% sulfuric acid and 4 ml of anthrone solution, and incubated for 15 minutes at 100 °C. After samples were cooled down, they were read against distilled water at 630 nm by spectrometer.

Total lipids were calculated by a slightly modified version of Bligh and Dyer's method [54]. Wet biomass containing 100 ± 5 mg was taken and 1.25 ml of chloroform and 2.5 ml of methanol were added. After 20 minutes of shaking, the samples were vortexed with 1.25 mL of chloroform. The samples were vortexed again by adding 1.25 ml of distilled water. After centrifuging the samples at 3000 rpm for 10 minutes, the lower phase was removed and the samples were evaporated at 70 ° C in a vacuum

oven. The glass containing lipid was kept 1 hour at 105 °C to reach constant weight. Triplicate samples were analyzed and the average values were taken.

Pigment contents were determined via centrifugation of 2 ml of microalgae cells of each strain at 5000 rpm for 5 min. Pellet was taken and suspended with 2 ml methanol (90%). The mixture was incubated in water bath at 80^{o}C for 5 min. The steps were continued by centrifugation at 10000 rpm for 5 min. The supernatant was transferred and measured by UV-Vis spectrophotometer at 470 nm, 665 nm and 655 nm against the solvent (methanol) blank. The concentration of chlorophyll a (chl-a), chlorophyll b and total carotenoids (car) were calculated as explained by Sumanta et al. (2014).

For Fatty Acid Methyl Ester (FAME) analyses, Laurens et al. (2014) and El-Shimi et al. (2014) procedures were followed for acid catalyzed in-situ transesterification with 5-10 mg of microalgal biomass. Methyl tridecanoate (C13: 0ME) was prepared as an internal standard and 20 µL C13:0 ME (10 mg / mL), 200 µL chloroform: methanol (2: 1, v / v), and 300 µL 0.6M HCl:methanol (methanolic hydrochloric acid) were added on 5- 10 mg dry algal samples and incubated for 1 hour at preheated 85 °C heat block. At the end of the incubation, 1 ml of hexane was added and the upper phase was measured by Gas Chromotography (GC). Shimadzu AOC-20i, GC 2010 model Gas Chromatography with CN100 capillary column (Teknokroma, Barcelona, Spain) with a length of 100 m × 0.25 mm and an internal diameter of 0.2 µm film thickness were used. The carrier gas was helium and the hydrogen gas flow was 40 ml / min whereas air gas flow was 400 ml / min.

4.3 Results and Discussion

4.3.1 Effect of iron on cell growth and biochemical composition

g The microalgal growth was monitored by measuring the Chlorophyll-a (Chl-a; mg L^{-1}) and Suspended Solid (SS) concentrations during the experiment due to the dark color of wastewater. Many studies also encountered the same problem where recent studies by Marazzi et al. (2017) and Huy et al. (2018) reported that measuring OD values for liquid digestate could be challenging due to their strong color.

Iron is an essential nutrient for the survival of all organisms. It is involved in chlorophyll biosynthesis, and it enhances biomass production. Therefore, iron deficiency invariably leads to a simultaneous loss of chlorophyll and degeneration of chlorophyll structure. In order to protect the light-harvesting pigment content,

including carotenoid, chlorophyll levels keep decreasing, where iron limited microalgae was reported to have lower pigment concentrations (Xing et al., 2007). In this study, the increase of iron concentration increased both *chl-a* and *car* amount until 3.1 mM $FeSO_4$ which supported the argument mentioned above. As depicted in Figure 4.1, for all iron concentrations, there were 7 days of slower growth for the mixed culture to adapt to the iron supplementation. On the other hand, the control batch had rapid growth since the mixed culture was adapted to ALD for almost 3 years.

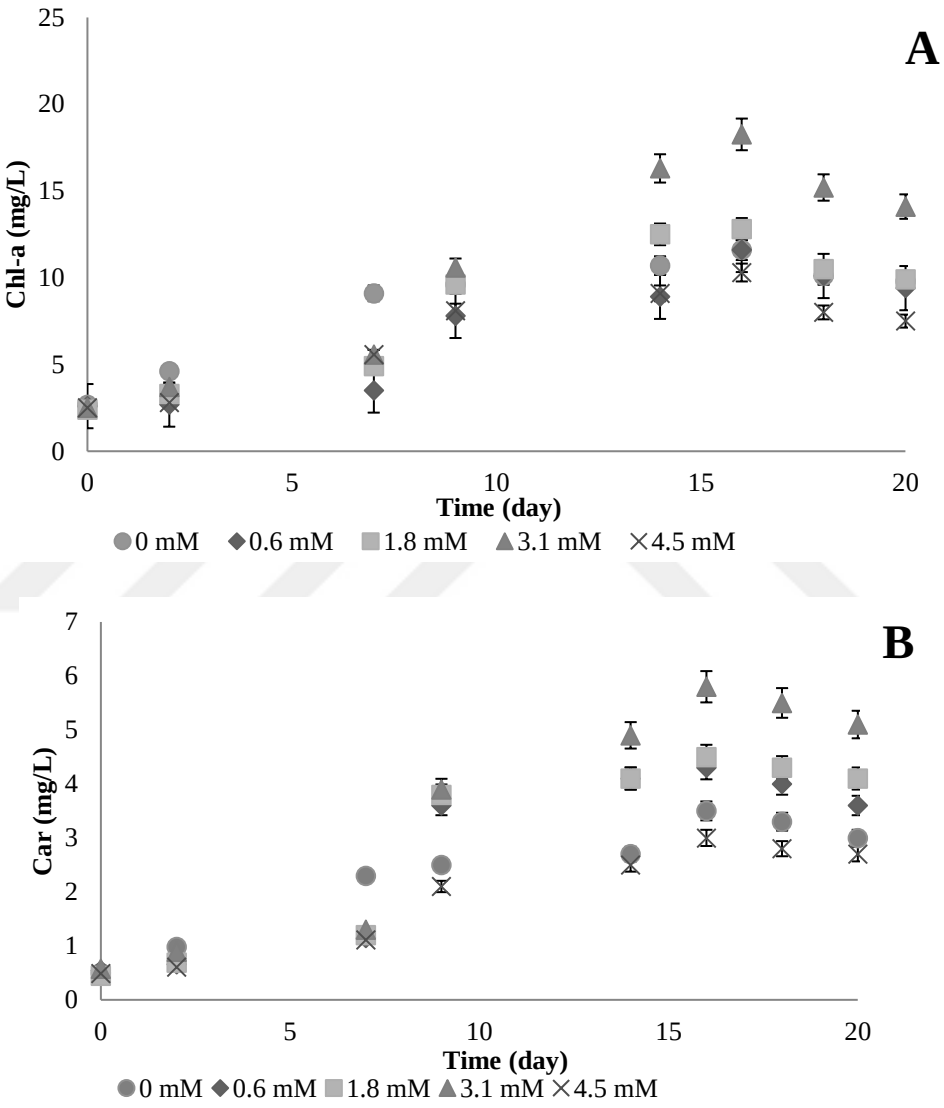

Figure 4.1 : Change in the concentration of (A) Chl-a, (B) Car as a function of time for different concentrations of iron.

After 7 days, rapid increase of *chl*-a was observed for all batches, and the batch with 3.1 mM $FeSO_4$ reached maximum *chl*-a amount (18.7 mg L^{-1}) after 16 days. For the carotenoid amount, also maximum amount was observed (5.8 mg L^{-1}) at 3.1 mM $FeSO_4$ concentration. These parameters indicated that the efficiency of photosynthesis

was related to iron nutrition as Morgan (1982) mentioned in their study. They explained that iron was also essential for cytochromes, ferredoxin and iron sulfur proteins and absence of it not only reduced the amount of these essential components of photosynthesis but also disrupted their optimal ultrastructural arrangement, which interfered with the function of the photosynthetic apparatus. However, 4.5 mM $FeSO_4$ concentration did not lead to any further increase of neither chl-a nor car concentrations (Figire 4-1). This can be due to the inhibition by iron-replete where excessive levels of essential metals, like iron, can be detrimental to the organisms (Shanab et al., 2012).

Compared with iron limitation studies, only a few experiments have been done under the iron replete conditions, and they were only with pure marine cultures grown in synthetic media. Sasireka & Muthuvelayudham (2015) applied various concentrations of $FeSO_4$ ranging from 10µM to 50µM on *Skeletonema costatum* and concluded that 30µM $FeSO_4.7H_2O$ resulted in the highest growth rate, which was a very low concentration of ferrous source compared to our study. Moreover, Huang et al. (2014) studied the highest final cell densities of three different algae *(Tetraselmis subcordiformis, Nannochloropsis oculata* and *Pavlova viridis*) in different concentrations (0.012, 0.12, 1.2 and 12 mM) of ferric ion, and by increasing the ferric ion concentrations from 1.2 to 12 mM, specific growth rates of three microalgae decreased significantly. In their study, the microalgal cultures treated with 0.12 mM iron showed the highest final cell densities. In our study, increasing ferrous concentration from 0 to 3.1 mM enhanced the growth of mixed culture; however, at 4.5 mM, the algal growth ceased. The algal biomass reached 1.06, 1.24, 1.35, 1.56 and 1.31 mg L^{-1} when the ferrous concentrations were 0.6, 1.8, 3.1, and 4.5 mM, respectively (Figure 4.2). Moreover, nutrient removal was correlated with the algal biomass productivity, whereas the highest nutrient removal for NH_3-N and PO_4-P was observed at 3.1 mM $FeSO_4$, with 89% and 76% removal efficiency, respectively.

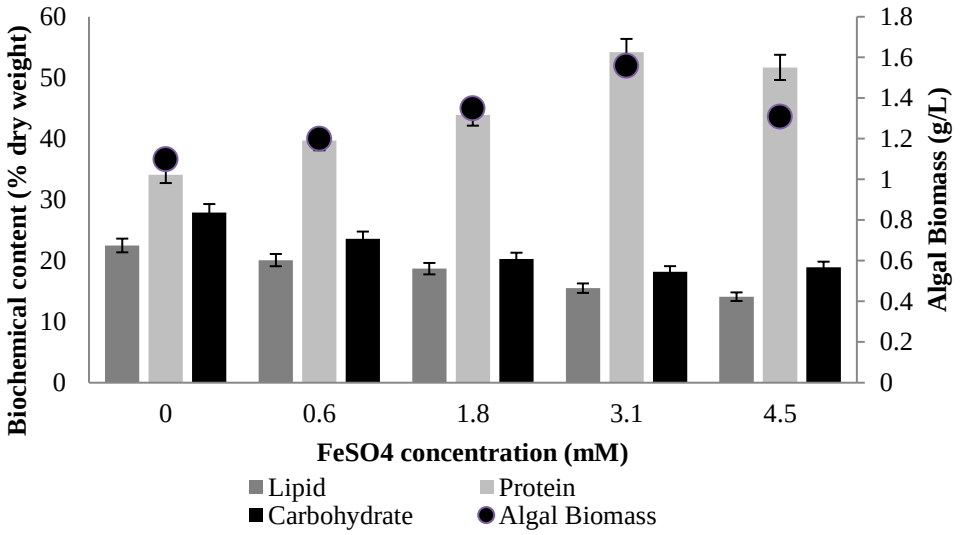

Figure 4.2 : Biochemical composition and biomass amount of the mixed culture at different $FeSO_4$ concentrations.

The growth and biochemical content of microalgae is important as a sustainable biological resource. The synthesis and accumulation of energy storage compounds can be enhanced by using appropriate variations in cultivation conditions. In this study, protein content increased from 34.1% to 54.2% when concentration of iron was increased at 3.1 mM $FeSO_4$ (Figure 4.2). In this study, along with an increase of protein content, the lipid and carbohydrate contents decreased simultaneously. The reason of concomitant decrease in the levels of both carbohydrates and lipids is that their synthesis pathways are related, where the energy is first stored in the form of carbohydrates and then the excess is converted into lipids (BenMoussa-Dahmen et al., 2016). The synthesis of triacylglyceride (TAG) and carbohydrate tends to compete with each other, and which one among those two types of energy storage is produced appears species specific (Rizwan et al., 2017). Liu et al. (2008) reported that *Chlorella vulgaris* supplemented with 10 µM iron exhibited a lipid content up to 56.6% of the biomass by dry weight, which was 3–7 fold higher than lower iron concentrations. Moreover, in Ghafari et al. (2018)'s study, *B. sudeticus*, *C. sorokiniana*, *C. vulgaris*, and *E. oleoabundans* showed 10, 60, 18, and 36% increase in lipid content at 4.32 µM Fe presence, respectively. This was expected as Fe increases overall neutral lipid accumulation due to the down-regulation of iron requiring fatty acid desaturase enzymes. In this study, lipid content decreased since dominancy in the culture changed in favor of a low-lipid-yield microalgae species. Overall, 3.1 mM ferrous addition into ALD increased the total protein content 60% along with highest biomass (1.56 g L^{-1})

and highest chlorophyll-a amount (18. mg L^{-1}). Therefore, 3.1 mM $FeSO_4$ addition was found to be the optimum iron concentration for a positive effect on algal biomass for further applications. However, in this study, mixed algal culture was analyzed rather than pure algal culture; therefore, the dominancy changes also affected the total biochemical composition of the system.

After the biochemical analyses of the mixed culture, the fatty acid compositions were determined. The composition of fatty acids of mixed culture was mainly Palmitic acid (C16:0) and Stearic acid (C18:0) for saturated fatty acids (SFU); Pentadecenoic acid (cis-10) (15:1), Elaidic acid (18:19t), Oleic acid (18:19c) and Gondoic acid 20:1 for monousaturated fatty acids (MUFA); and Linolelaidic acid (18:2 9t), Linoleic acid (18:2 9c), α-Linolenic acid (18:3) and Eicosadienic acid (20:3) for polyunsaturated fatty acids (PUFA); which were the most common fatty acid profiles of algae. The highest amount of Palmitic acid (C16:0) and Stearic acid (C18:0) were observed at 3.1 mM $FeSO_4$ concentration with 46.6% and 8.3%, respectively. As 4.5 mM $FeSO_4$ and control cultivation showed similar results for growth; the fatty acid composition concentrations had also similarities. Both of them had C18:1 n-9c (oleic acid), whereas other concentrations did not have C18:1 n-9c which is the most representative fatty acid of MUFA mainly containing in olive oil. Moreover, C20:3 disappeared at the iron concentrations of 3.1 and 4.5 mM, which indicates that different iron concentrations could affect the composition of the fatty acids and can be effectually altered by changing iron concentrations. Since the unsaturation grade affects the cold flow, stability and ignition quality of diesel fuel (Rizwan et al., 2017), C18:3 amount was limited as < 12% (w/w) by European biodiesel standard EN14214 (Pandit et al., 2017). According to the results from this study, C18:3 amount was measured between 1.3-3.1%, which examined the high quality of the biodiesel (Table 4.1).

Table 4.1 : FAME profiles for mixed microalgae under different iron concentrations.

Fatty Acids	$FeSO_4$ Concentration (mg L^{-1}) (Mean±SD*), n=3				
	0[a]	130[b]	400[c]	700[d]	1000[e]
	Fatty Acid Composition (%, w/w)				
Saturated fatty acids (SFU)					
16:0	*32.4±2.9*	**39.5±2.2**	**44.3±3.2**	**46.6±5.5**	**41.6±3.4**
18:0	*4.7±0.22*	**5.6±0.25**	**6.7±0.21**	**8.3±0.1**	**6.1±0.08**
Monounsaturated fatty acids (MUFA)					
15:1	*6.6±0.34*	**12.6±1.56**	**8.8±0.99**	**10.5±0.5**	**13.4±0.27**
18:1 n-9t	*10.1±1.5*	**21.6±3.4**	15.7±3.4	**23.1±0.34**	-
18:1 n-9c	*7.6±0.44*	-	-	-	**9.2±0.05**
20:1	-	-	**1.8±0.5**	-	-
Polyunsaturated fatty acids (PUFA)					
18:2 9t	*4.1±0.11*	4.6±0.33	-	4.1±0.09	**3.8±0.11**
18:2 9c	*6.8±0.23*	**11.5±0.95**	**10.2±0.1**	6.4±0.35	**16.6±036**
18:3	*1.3±0.09*	**2.1±0.06**	**3.1±0.12**	**2.8±0.05**	**1.5±0.04**
20:3	*0.26±0.01*	**2.4±0.08**	**6.4±0.45**	-	-

*SD: Standard Deviation

Bold font indicates statistically significant difference with respect to 0 mg L^{-1} Fe concentration (t-test, $P<0.05$).

4.3.2 Effect of magnesium on cell growth and biochemical composition

Since magnesium is the center element of chlorophyll, a higher chlorophyll-a amount was expected with increasing $MgSO_4$ concentrations; however, there was no significant differences on neither biomass amount nor chlorophyll amount (Table 4.2). However, the highest carotenoid amount between all batches and concentrations was observed at 0.4 mM $MgSO_4$ concentration (7.4 mg L^{-1}), where it was 3.3 mg L^{-1} at control cultivation and 5.8 mgL^{-1} at 5.1 mM $MgSO_4$ presence. Carotenoids are synthesized in the chloroplast by the action of a series of nuclear-encoded membrane proteins, and Varela et al. (2015) mentioned that some microalgae have the ability to accumulate carotenoids under unfavourable conditions.

Table 4.2 : Impact of different magnesium concentrations on cell growth, nutrient removal efficiencies, and pigments.

$MgSO_4$ (mM)	Algal biomass[a] ($g\ L^{-1}$)	Chlorophyll-a[b] ($mg\ L^{-1}$)	Total Carotenoid[c] ($mg\ L^{-1}$)	PO_4-P removal[d] (%)	NH_3-N removal[e] (%)
(Mean±SD*), n=3					
Control	*1.22±0.25*	*18.5±0.9*	*3.3±0.45*	*75.1±5.5*	*85.7±7.6*
0.4	1.36±0.28	**15.4±1.1**	**7.4±0.9**	69.2±4.1	88.3±9.1
2	1.02±0.15	**14.4±1.3**	**6.6±0.85**	**65.2±3.6**	85.2±9.6
3.5	1.1±0.20	**12.7±1.1**	**6.1±0.8**	66.7±4.9	85.6±10.5
5.1	1.15±0.19	**11.1±0.8**	**5.8±0.71**	**55.2±3.9**	78.1±8.8

*SD: Standard Deviation

Bold font indicates statistically significant difference with respect to control $MgSO_4$ concentration (t-test, $P<0.05$).

The highest nutrient removal for NH_3-N and PO_4-P were observed at 0.4 mM $MgSO_4$ with 88.3% and 69.2% removal efficiency, respectively. Magnesium is one of the chemicals being studied due to its potential for P and N removal in municipal wastewater treatment. Its reaction mechanism is the same as other chemical precipitation processes (Liang, 2007). Moreover, L. Huang et al. (2014) reported that Mg^{2+} dominates algal cell absorption and phosphorus utilization. However, in this study, magnesium addition did not improve neither nitrogen nor phosphorus removal compared to control and iron cultivation batches.

The biochemical components of microalgae can be also influenced by the different concentrations of magnesium in the culture medium. However, in this study there were fluctuations between Mg concentration and biochemical composition. The lipid amount increased by 27% when the magnesium concentration was increased from 0 mM to 3.5 mM; however, it started to decrease at 5.1 mM $MgSO_4$ presence. Huang et al. (2014) also observed that the supplementation of 100 μM Mg^{2+} to the culture medium increased the lipid content of *Monoraphidium* sp. This result indicated that Mg^{2+} has the potential to stimulate lipid accumulation in microalgae but the threshold of the concentration where it starts to cause inhibition should be determined. Ulloa et al. (2012) and Sydney et al. (2011) also mentioned that the addition of an appropriate concentration of Mg^{2+} to the culture medium increases the lipid content and lipid productivity of microalgal cells, whereas excess Mg^{2+} decreases the lipid content and lipid productivity (Figure 4.3).

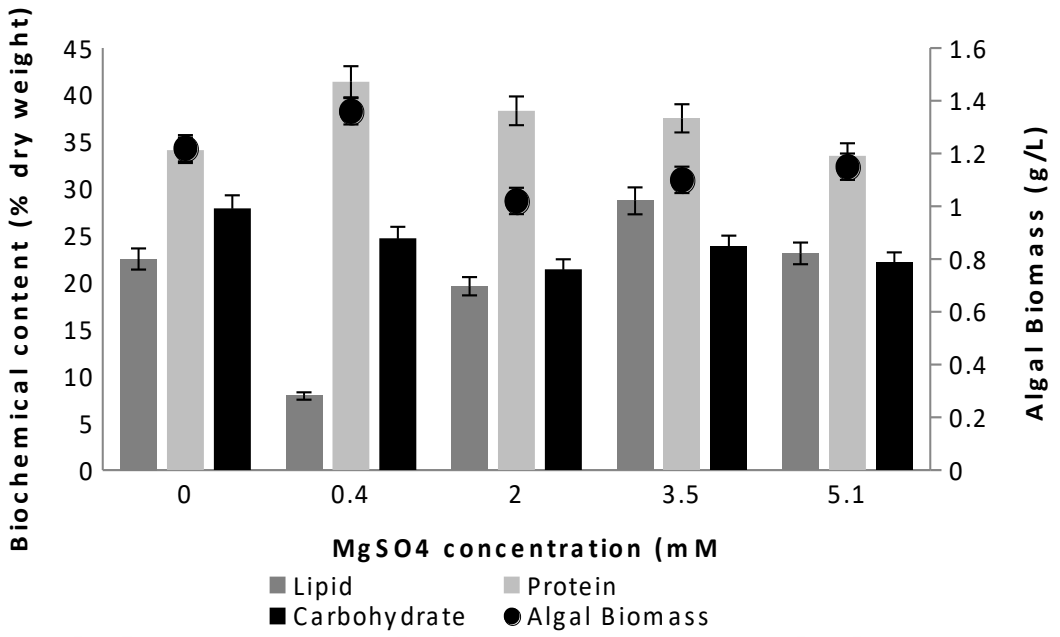

Figure 4.3 : Biochemical composition and biomass amount of the mixed culture at different $MgSO_4$ concentrations.

The fatty acid compositions mainly consisted of C16:0, C18:0, C18:1 and C18:2; however, Mg^{2+} addition decreased the amount of fatty acids under all concentrations. The highest C16:0 was observed at control with 176.2 ppm, where it was 55.1, 43.2, 42.6 and 27.5 ppm for 0.4, 2, 3.5 and 5.1 mM $MgSO_4$ concentrations, respectively (Table 4.3).

Table 4.3 : Fatty acid compositions under different Mg concentrations.

	Fatty acid compositions (ppm) (Mean±SD*), n=3			
$MgSO_4$ concentration (mM)	C16:0	C18:0	C18:1n9c	C18:2 9c
Control	*176.2±12.1*	*19.09±2.5*	*37.88±5.99*	*55±6.23*
0.4	**55.1±3.5**	**3.8±0.44**	33±4.35	**22.5±3.22**
2	**43.2±5.8**	**2.36±0.18**	29.64±4.12	**17.76±1.98**
3.5	**42.6±4.7**	**0.86±0.05**	**20.1±3.11**	**14.8±2.12**
5.1	**27.5±3.3**	**0.55±0.04**	**5.6±0.76**	**12.4±1.99**

*SD: Standard Deviation

Bold font indicates statistically significant difference with respect to control $MgSO_4$ concentration (t-test, $P<0.05$).

Addition of 0.4 mM $MgSO_4$ to ALD was found to be the most convenient concentration for mixed culture due to higher biomass and higher carotenoid amount compared to the control batch.

4.3.3 Effect of Iron and Magnesium on Microbial Composition

Algal microbiome of mixed culture was measured for the optimum concentration of iron and magnesium, and compared with the control growth. Multi-marker metabarcoding approach was used for microbial community analysis by analyzing four different markers: 16S rRNA, 23S chloroplast rRNA, 18S rRNA and tufA. Photosynthetic microorganisms like cyanobacteria and microalgae have been considered important in the production of valuable co-products along with biofuels in an economically effective and environmentally sustainable way by improving their high value products (Parmar et al., 2011). In our mixed algae cultures, *Diphylleia rotans, Synechocystis PCC-6803* and *Chlorella sorokiniana* were found to be mostly abundant species, as confirmed by 18S rRNA, 16S rRNA, 23S chloroplast rRNA and tufA marker analyses. When iron supplementation was applied on the mixed algal culture, the abundances of the same dominant species were diminished or increased. The abundance of *Synechocystis PCC-6803* and *Chlorella sorokiniana* increased approximately 1.4 folds and 2,5 folds, respectively, for both 16S and 23S rRNA, while *Diphylleia rotans* abundance did not change noticeably. Most abundant microorganisms detected in the mixed cultures were shown in Table 4.4, and their phylogenetic trees are given in Figure A2.

The aim of metabarcoding is not only characterizing the communities and biodiversity with high sensitivity, but also detecting the community dynamics, including the interactions between microalgae and other microorganisms. Some studies indicated that presence of bacteria in mixed algal culture can increase algal production and microalgal-bacterial interactions may lead to increased levels of microalgae species and algal production of valuable compounds (Fuentes et al., 2016). Bacteria relationships on algal growth can be mutualistic or parasitic, and knowledge of these mechanisms can be used in order to enhance the algal biomass and high value products (Afi et al., 1996; Choix et al., 2012).

Table 4.4 : Most abundant microorganisms (>2% abundance) in the cultures based on the taxonomic classification by QIIME 2 analysis.

Marker genes	Genus / species	% abundance		
		Control	Iron Batch	Magnesium Batch
16S rRNA	*Synechocystis PCC-6803*	**20.14**	**27.51**	**20.02**
	Cyanobium PCC-6307	**5.75**	0.07	0.07
	Chlorella sorokiniana	**1.79**	**3.93**	**2.84**
	Dechloromonas fungiphilus	**3.14**	0.01	0.01
	Burkholderiaceae	**2.55**	0.84	**3.86**
	Desulfovibrio oxamicus	**2.00**	0.01	0.03
	Thauera	0.00	**3.28**	0.94
	Azospirillum	**5.02**	0.01	0.03
	Burkholderiaceae	**2.55**	0.84	**3.86**
	Thermomonasfusca	0.85	0.01	**3.21**
	Phycisphaeraceae SM1A02	0.10	**2.39**	0.08
	Planctomicrobium piriforme	0.54	**2.28**	1.91
	Turneriella	0.19	0.89	**4.07**
	Microtrichaceae IMCC26207	0.09	**3.16**	0.31
	Sediminibacterium	0.06	0.27	**2.30**
	Gemmatimonas	**2.18**	1.14	1.04
18S rRNA	*Diphylleia rotans*	**45.54**	**42.17**	**2.02**
	Trebouxiophyceae	**10.14**	**19.55**	**12.03**
	Leptophryidae sp. *WaAra*	**7.60**	0.05	0.04
	Cercozoa sp. *1 YG-2013*	**6.57**	0.18	**7.06**
	Fungi	**4.64**	0.03	0.02
	Poterioochromonas malhamensis	**3.65**	0.02	0.02
	Characium saccatum	**2.66**	0.24	**6.62**
	Poteriospumella	**1.92**	0.02	0.00
	Spongomonas	1.58	**7.52**	1.27
	Nuclearia	0.25	**4.91**	1.36
	Amoebozoa sp. *Pa18*	0.40	0.32	**18.17**
	Chlamydomonas noctigama	0.12	0.19	**18.11**
	Paraphysoderma sedebokerense	0.84	0.49	**13.22**
23S rRNA	*Synechocystis PCC-6803*	**59.69**	**81.85**	**80.71**
	Cyanobium PCC-6307	**28.64**	0.32	0.39
	Chlorella sorokiniana	**2.18**	**5.95**	**5.93**
	Planctomicrobium piriforme	0.33	**5.34**	**4.71**
tufA	*s_Chlorella sorokiniana*	**26.72**	**59.09**	**31.23**
	c_Chlorophyceae	**3.85**	**32.16**	**38.06**

The algae-bacteria microbiome can have key roles for modulating microalgal populations by promoting microalgae growth. In 16S rRNA analysis, *Synechocystis PCC-6803* was identified in the control sample with highest abundance (20.14%), similar to an abundance of 20% for the same species in the magnesium added culture. This demonstrated that magnesium had no effect on the dominancy *of Synechocystis PCC-6803*. However, with iron addition, the abundance of *Synechocystis PCC-6803* increased up to 27.51%. This increase was reflected on the protein amount of the mixed culture since *Synechocystis PCC-6803* has high protein content (65%) (Touloupakis et al., 2016). Another cyanobacteria *Cyanobium PCC-6307* was identified in the control culture with an abundance of 5.75%; however, with iron and magnesium addition, it was not able to survive at all. Moreover, *Chlorella sorokiniana* was present in the control sample with 3.5% abundance and the dominancy increased more than twice, up to 8.46% when iron was added; and up to 5.26% when magnesium was added. This increase was also reflected on the protein amount of the mixed culture since the dry weight analysis of *C. sorokiniana* shows that it has 40% protein, 30–38% carbohydrate and 18–22% lipid content (Lizzul et al., 2018). Moreover, bacterial species belonging to *Proteobacteria, Spirochaetes, Actinobacteria, Bacteroidetes* and *Gemmatimonadetes* phyla had different abundance in control, magnesium and iron added conditions. The total abundance of proteobacteria in the control sample was 43% approximately, whereas this abundance decreased to approximately 32% and 40% for the mixed culture when iron and magnesium was added, respectively. In the control culture, the abundance of *Azosprillium* bacteria was 5%, *Dechloromonas fungiphilus* species was 3.14%, *Desulfovibrio oxamicus* specie was 2%, *Burkholderiaceae* bacteria was 2.55% and *Gemmatimonas* bacteria was 2.18%. These most abundant bacteria species found in the mixed culture belong mostly to *Proteobacteria* family, which indicated that algae-proteobacteria interactions were dominated in control environment when there were neither iron nor magnesium addition. In the iron added environment, the abundance of *Proteobacteria* species decreased, leaving *Thauera* species to be present in 3.28%, which was not detected in the control sample. Moreover, *Planctomycetes* phyla of bacteria had been found in iron-added environment together with *Phycisphaeraceae* and *Planctomicrobium* bacteria with 2.39% and 2.28% abundances, respectively. In addition, one *Actinobacteria* phylum bacteria belonging to *Microtrichaceae* family has emerged with 3.16% abundance in the iron added environment. The amount of *Proteobacteria*

was higher in the magnesium added environment, where *Thermomonas fusca* species have adapted to the environment and survived with 3.21% abundance. The two *Planctomycetes* bacteria that emerged under iron stress was not detected under magnesium stress with 16S rRNA analysis, however with 23S rRNA analysis, *Planctomicrobium piriforme* bacteria was detected as abundant as in iron stress cultures. In addition, *Turneriella* genus of *Spirochaetes* was found to be abundant in magnesium stress culture with 4.07% and *Sediminibacterium* genus of *Bacteroidetes* was abundant with 2.3%.

In 18S rRNA analysis, heterotrophic flagellate *Diphylleia rotans* was identified as the most dominant algae species in both control sample and iron added sample, with abundances of 45.54% and 42.17% respectively. However, the dominance was diminished in magnesium added culture to an abundance of 2.02%, which clearly demonstrated that magnesium had a negative effect on *Diphylleia rotans*. On the other hand, *Chlamydomonas noctigama* emerged with 18.11% abundance, *Amoebozoa* sp. *Pa18* species emerged with 18.17% and also a fungi species, *Paraphysoderma sedebokerense,* emerged with 13.22% abundance in response to magnesium addition. These results concluded that macro elements such as iron and magnesium could not only affect the concortia of mixed cultures but also wiped out or emerge microorganisms facultatively. *Poterioochromonas malhamensis*, which is known to be a microalgal predator (Ma et al., 2018) was detected only in the control culture. *Poterioochromonas malhamensis* can feed on *Chlorella* sp., which was consistent with the low amount of *Chlorella* sp. in the control sample. Therefore, one of the reasons of the increase in the dominance of *Chlorella sorokiniana* species in different environmental stress conditions might be the disappearance of *P. malhamensis* species. *Characium saccatum* is another green algae species, which was detected with 2.66% abundance in the control culture and increased up to 6.62% with magnesium addition. However, the abundance of *Characium saccatum* decreased to almost zero with iron addition. Different amoeboid organisms were also detected in 18S rRNA analysis such as *Leptophryidae* sp. *WaAra* were detected with 7.6% dominance in only control sample, while an unclassified *Cercozoa* sp. *1 YG-2013* was detected both in the control culture and magnesium added culture with 6.57% and 7.06% abundances, respectively; which implied a negative effect of iron on *Cercozoa* sp. *1 YG-2013*. Flagellated protozoa *Spongomonas* genus and *Nuclearia* genus were detected with highest abundance in the iron added culture, whereas it was very low at control and

magnesium added samples, which indicated a positive effect of iron on *Spongomonas* genus and *Nuclearia* genus.

23S rRNA analysis identified *Synechocystis PCC-6803* as the dominant species for all conditions with the same amount of increase when iron and magnesium were added. However, the second dominant species *Cyanobium PCC-6307* disappeared when iron and magnesium was added as it was also observed in 16S rRNA results which supported the accuracy of results between different markers.

In tufA analysis, a curated database specialized to detect algal species was used (Sauvage et al., 2016) which identified the green algae *Chlorella sorokiniana* as the most dominant algal species with 26.7, 59, and 31.2% relative abundance for control, iron and magnesium batch, respectively.

In conclusion, the effect of iron and magnesium replete on mixed culture can be explained as the acceleration of the growth of protein-rich species, such as *Chlorella sorokiniana,* due to the higher need of protein synthesis, leading to improve high protein content of the total algal biomass. Moreover, iron and magnesium addition did not increase neither lipid nor carbohydrate and they were both the highest at control batch (Figure 4.2 and Figure 4.3). According to 23S rRNA results, At control batch *Cyanobium PCC-6307* relative abundance was 28.64%, where it was 0.32% and 0.39% when iron and magnesium were added, respectively. This big difference on relative abundance of *Cyanobium PCC-6307* in different growth media clarified the reason of higher amount of lipid and carbohydrate observed at control batch comparing to other batches.

4.3.4 Principal component analysis

Principal component analysis (PCA), which is useful for discerning patterns within the species viability data itself, was applied to observe the relation between the measured parameters and the dominant species in different growth conditions.

The PCA components for each culturing condition were plotted in relation to the biochemical composition, pigment composition and relative abundances of microbial species detected in highest amount via each of 16S, 18S and tufA marker analysis. Mostly abundant cyanobacteria Synechocystis PCC-6803 and Cyanobium PCC-6307, heterotrophic flagellate Diphylleia rotans, green microalgae Chlamydomonas noctigama and Chlorella sorokiniana were included in the PCA biplot (Figure 4.4).

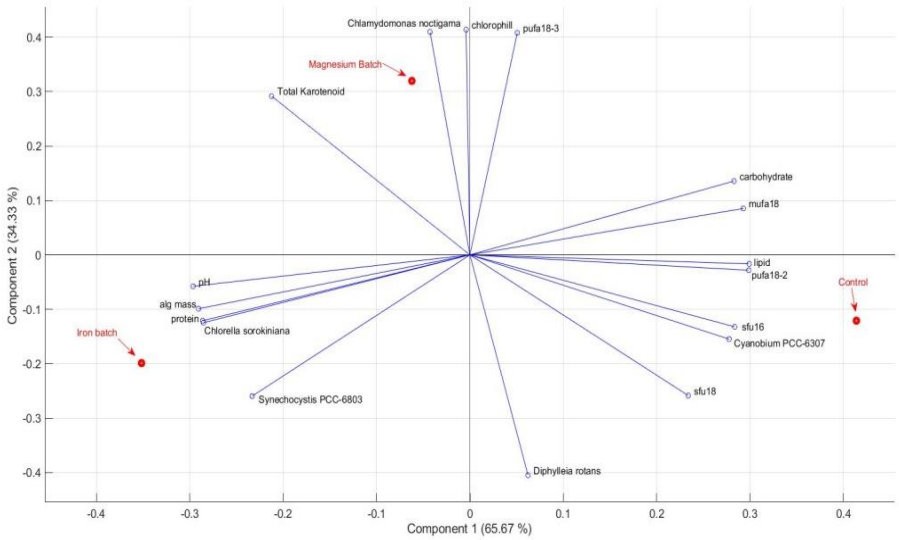

Figure 4.4 : PCA biplot for various parameters measured for the mixed culture with different growth media conditions.

Iron effect on the mixed culture was shown to be correlated with the protein content as well as with the abundances of *Chlorella sorokiniana* and *Synechocystis PCC-6803,* which are both protein-rich species. Magnesium effect, on the other hand, was shown to be mostly correlated with the pigments as well as with the abundance of *Chlamydomonas noctigama.* The control batch was correlated with the lipid content as well as with the abundance of *Cyanobium PCC-6307*. The fatty acid (FA) compositions of the mixed cultures were dependent on the taxonomic abundances and cultivation conditions. PCA identified the abundance of *Cyanobium PCC-6307* as the major source of variability within the control culture. This variability could be mostly explained by the differences in SFU (C16, C18), MUFA (C18:1), and PUFA (C18:2) contents. C18:3 content was shown to be increased in magnesium batch, which was also observed as positively correlated with the magnesium batch sample in the PCA biplot (Figure 4.3). Poerschmann et al. (2004) also reported that polyunsaturated fatty acids, especially linolenic acid (18:3), was the most abundant in their study with *Chlamydomonas* sp.

4.4 Conclusion

Successful cultivation of microalgae on wastewater, particularly on digestate, requires close monitoring since each wastewater has its own characteristics. In this study, the microbial community profiles and dynamics were first identified via metabarcoding of 16S rRNA, 18S rRNA, 23S chloroplast rRNA an tufA regions. The community

profiles changed drastically due to the macro element addition where the differences in the mixed algal community can be helpful for the adaptation to different environmental and growth conditions. 3.1 mM $FeSO_4$ and 0.4 mM $MgSO_4$ addition was found to be the optimum concentration with a positive effect on growth and biochemical composition. Moreover, the dynamics of undefined algal microbiome grown in anaerobic digestate showed significant changes and demonstrated how symbiotic life can be changed if macro elements were added to the ALD. Therefore, this algal microbiome might be a solution for both reducing adverse effect of anaerobic liquid digestate and lowering the cost of microalgae production to help commercialisation of algae-derived products.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 General Conclusion

In this thesis, it was successfully demonstrated that ALD can be used as the nutrient rich cultivation medium for mixed microalgae culture with high growth rates, despite the fact that liquid digestate is not the ideal growth media for microalgae due to its high ammonia content, dark color, and particulate matter. Working with mixed microalgal culture reduced the risk of system contamination compared to working with pure culture which generated the operation in difficult conditions easier by changing its dominance and increase the process feasibility.

However, successful cultivation of mixed microalgae on on digestate, requires close monitoring since each wastewater has its own characteristics. Therefore, in this thesis, the microbial community profiles and dynamics were identified via metabarcoding of 16S rRNA, 18S rRNA, 23S chloroplast rRNA an tufA regions. The community profiles changed drastically due to the macro element addition (iron, magnesium, sodium chloride) where the differences in the mixed algal community can be helpful for the adaptation to different environmental and growth conditions. Moreover, the dynamics of undefined algal microbiome grown in anaerobic digestate showed significant changes and demonstrated how symbiotic life can be changed if macro elements were added to the ALD. Moreover, salt stress is the limitation of invasive or competing microorganisms with microalgae which is crucial when the cultivation media is unsterilized wastewater as in real scale studies. This thesis provided the cost-effective strategy towards the cultivation of mixed microalgae consortia, which could be further converted in to biofuels along with the digestate treatment. It was observed that mixed microalgal culture could be able to perform better than pure culture by changing species dominance and/or biochemical compositions due to stress conditions caused by not only dark, high ammonicial and particulate rich digestate; but also the salinity; therefore, reduced the risk of system contamination and increase the process feasibility.

In conclusion, in this thesis, the production of microalgae biomass have been successfully carried out, and the macronutrients needed in the production of microalgae have been provided from the digestate effluent obtained from a real-scale anaerobic digester using a mixture of domestic organic solid waste, industrial treatment sludge and animal feces. Moreover, it helped to understand how the dynamics of symbiotic life changes if macro elements are added to the ALD and reveal that microalgae can adapt to adverse environmental conditions by fostering the diversity with a positive effect on high value product. This thesis might help to be a solution for targeting both reducing adverse effect of anaerobic liquid digestate by mixed microalgae and increasing product values by valorization and give an idea that digestate and/or other wastewaters can be diluted with sea water instead of tap water in the future studies.

Moreover, by this thesis, it was proves that digestate cultivated algae are rich source of primary (carbohydrates, proteins, lipids) and secondary (pigments) metabolites that could be exploited to produce biofuels, bio-polymers, biofertilizers, nutraceuticals, food/health grade compounds, enzymes, feed supplements etc. and the treated digestate can be reutilized for the agricultural or industrial purposes according to the high removal rates by mixed culture.

5.2 Challenges and future recommendations

The ability of microalgae to absorb additional nutrients from the environment has been fully researched in last 70 years. Apart from the fertilizer or amendment properties of digestate, nowadays there are some other methods to utilize it. These new methods are very creative and make the possibility of proper utilization of digestates with different quality. Even though high interest has been observed in the treatment of digestate effluent by algal cultivation, unspecified reasons that affect this practice include microalgal species, microorganisms from anaerobic digestion, geographical and seasonal variation, costs, recycling concerns and policy implications.

In this regard, a biorefinery approach is feasible where a spectrum of marketable products may be obtained from the single process. Moreover, the nutrient removal efficiency of microalgae can be enhanced by applying binary cultures that may consist of microalgae-microalgae, or microalgae-bacteria associations. These associations also improve the flocculation ability while avoiding the contamination chances and

may improvise the natural metabolite content of microalgae. However, detailed studies are required in the future to study the impact of digestate-derived stress on the biomass productivity of consortia and the post-harvesting processing of the biomass along with the metabolite content. Moreover, stress-responsive genes should be identified in the future which would be later targeted to engineer the selected microalgal strains to achieving industrial robustness in algal biorefineries.

Amongst the many thousands of microalgal species present in nature, there are only a few commonly occurring species currently studied and known to be robust survivors in digestate. These include species belonging to the genera *Chlorella*, *Scenedesmus*, and *Desmodesmus*, with key species being *Chlorella vulgaris* and *Scenedesmus obliquus*. However, mixed algal consortia and/or algal-bacteria consortia are more suitable for large-scale cultivation on wastewater than unicellular culture, by acting symbiotically, especially in terms of preventing contamination and enabling long-term cultivation. A key challenge in mixed consortia is to ensure that bacteria do not dominate the consortia system causing the algal cells to crash. Another challenge in large-scale algal cultivation on digestate is the dynamic nature of the algal-bacterial consortia. The processing of microalgal biomass into products is expensive. If a multi-product approach is adopted for the algal biorefinery, it would be more competitive with other biomass options. It should be required to conduct detailed studies to elucidate the technical and economical impact of using residual algal biomass for traditional applications in energy, environment and agriculture sectors after extracting the high-value products.

Undeniably, the environmental, economical, social and cultural benefits involved in microalgae mass production have the potential to make significant contributions to a sustainable industry. However, in order to develop and commercialise a sustainable product in the long term, the associated risks (which commonly exist in production processes) must be known and addressed through relevant measures. Efficient government policies, proactive company behaviours and positive public participation will play an important role in tackling, or even eliminating, potential risks associated with the algal biofuel industry. Only in this way, can the algal industry enjoy prosperity in a sustainable manner.

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APPENDICES

APPENDIX A : Chapter 3 supplementary material

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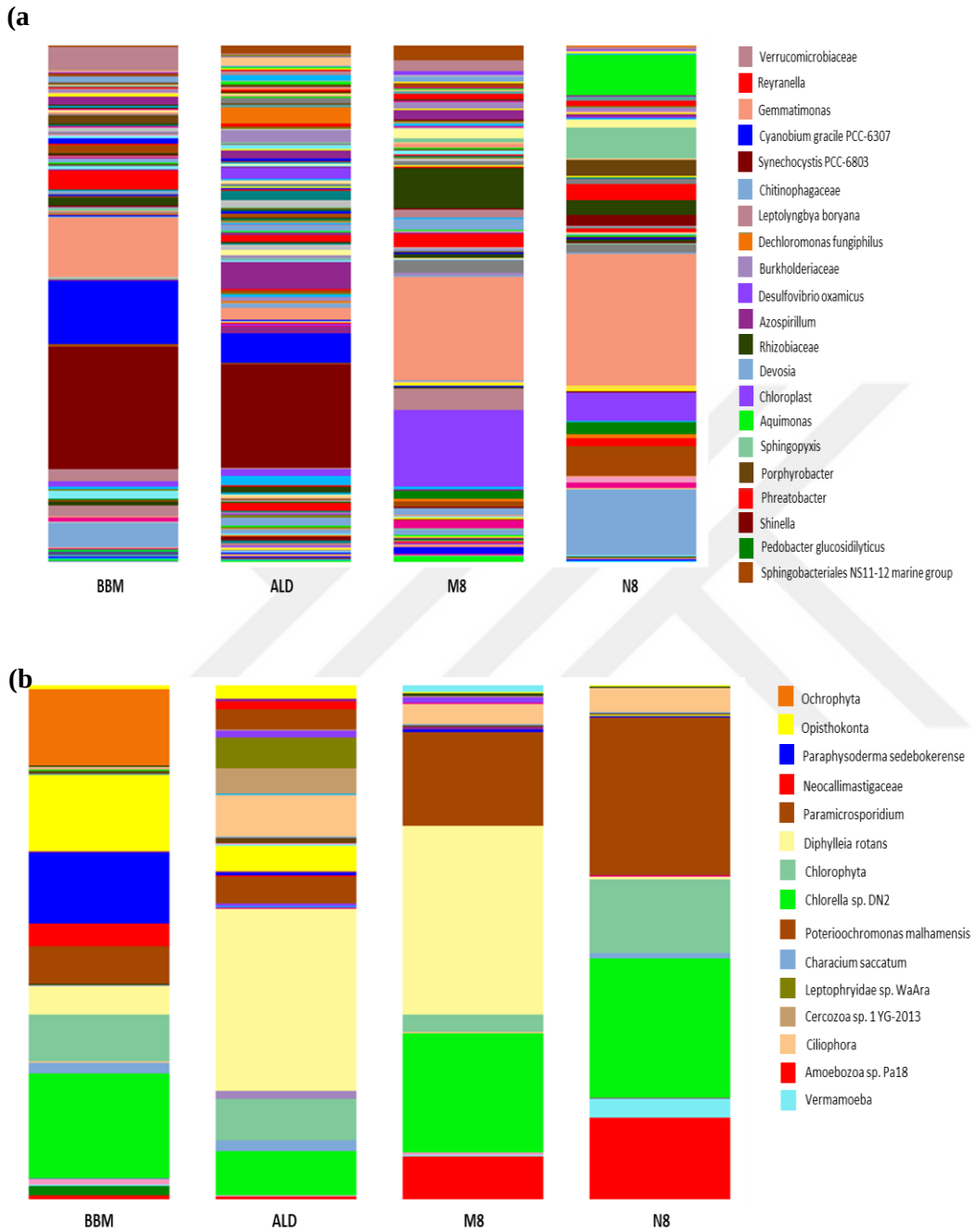
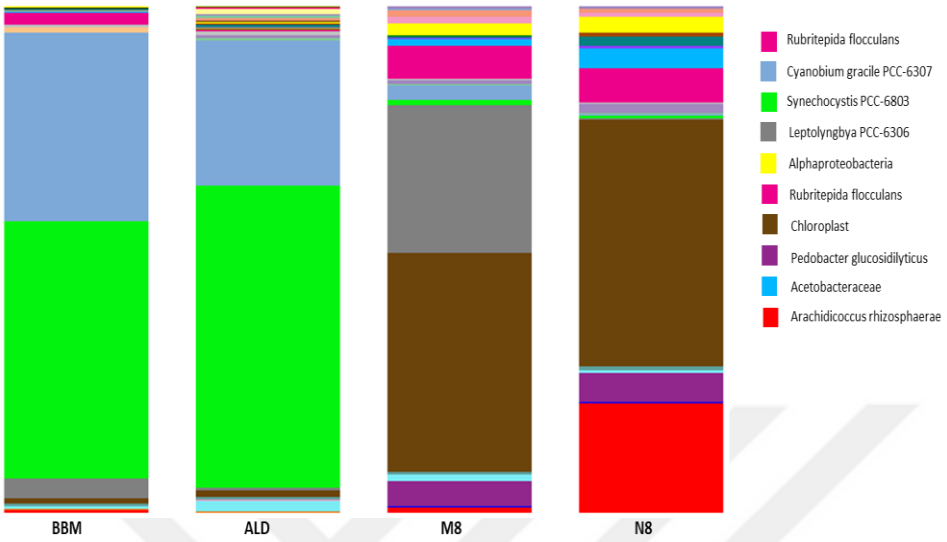


Figure A. 1 : Bar plots showing variation in the relative abundances of bacterial taxonomy in mixed microalgae microbial communities (BBM, ALD, M8 and N8). Colors represent microbial taxonomy classified by Silva taxonomy (release_132) with using (a) 16S rDNA marker regions, (b) 18S rDNA marker regions and (c) 23S rDNA marker regions, by (d) tufA database

(c)



(d

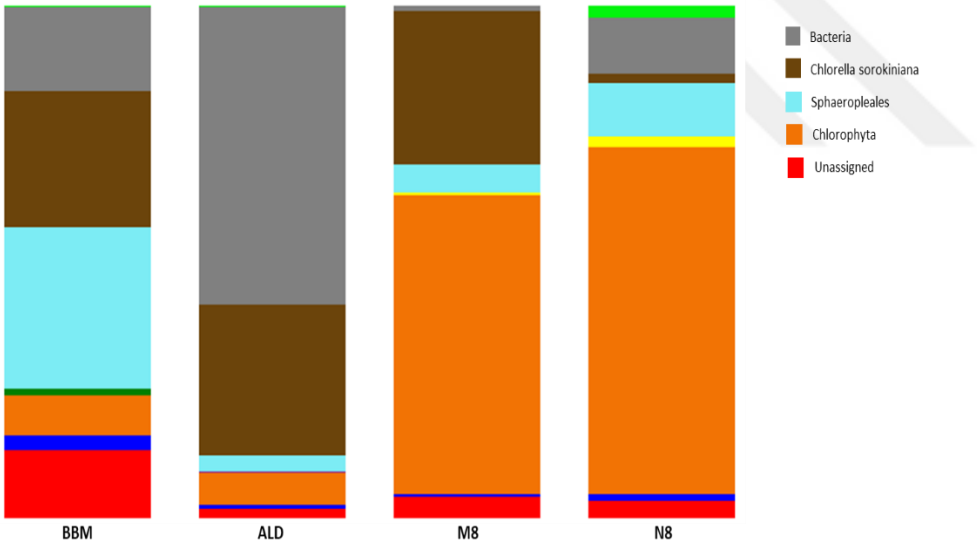


Figure A. 2 (continued): Bar plots showing variation in the relative abundances of bacterial taxonomy in mixed microalgae microbial communities (BBM, ALD, M8 and N8). Colors represent microbial taxonomy classified by Silva taxonomy (release_132) with using (a) 16S rDNA marker regions, (b) 18S rDNA marker regions and (c) 23S rDNA marker regions, by (d) tufA database.

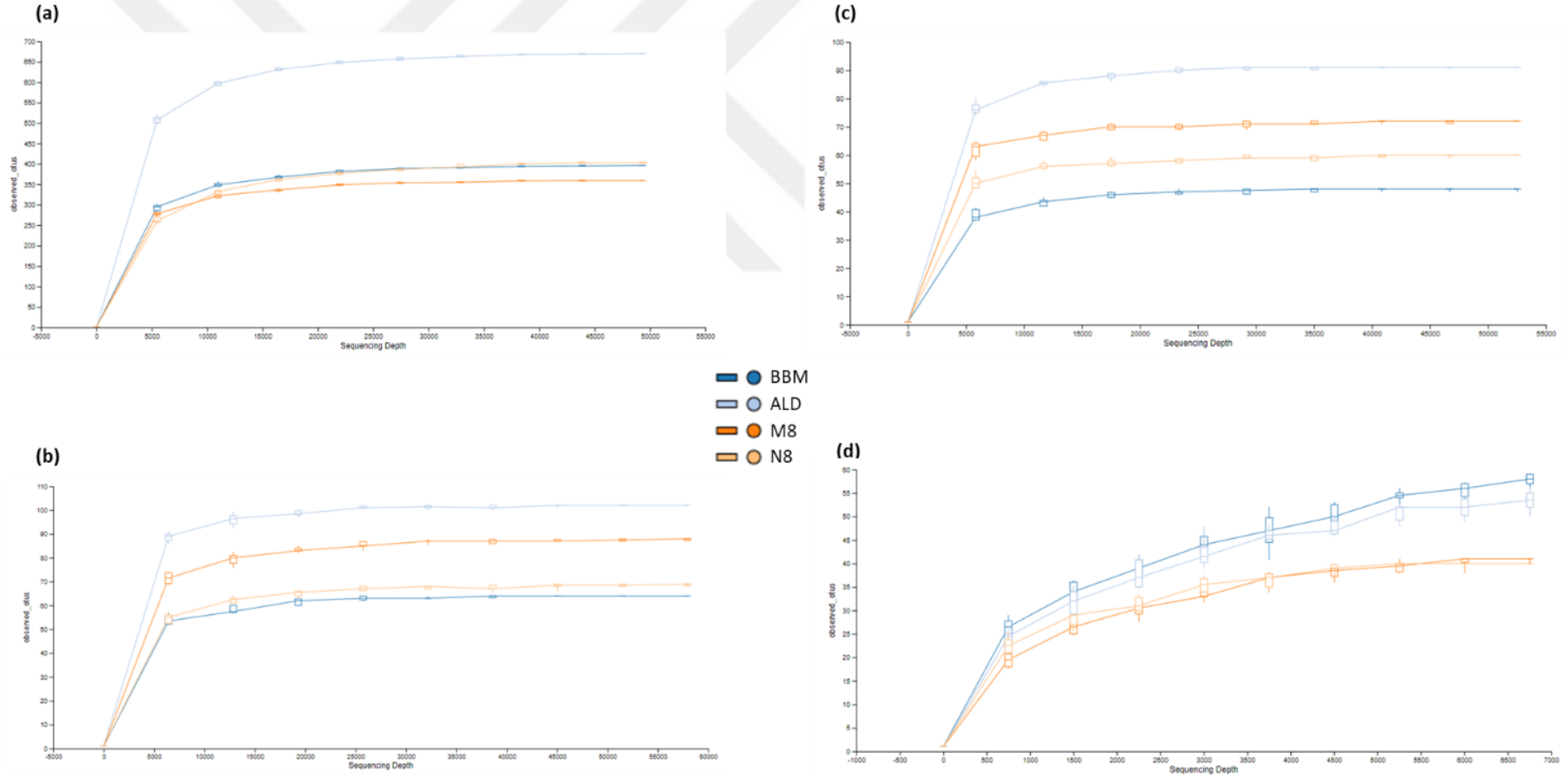


Figure A. 3 : Rarefaction plots of the Alpha diversity of all samples for (a) 16S rRNA, (b) 18S rRNA, (c) 23S rRNA and (d) tufA marker regions.

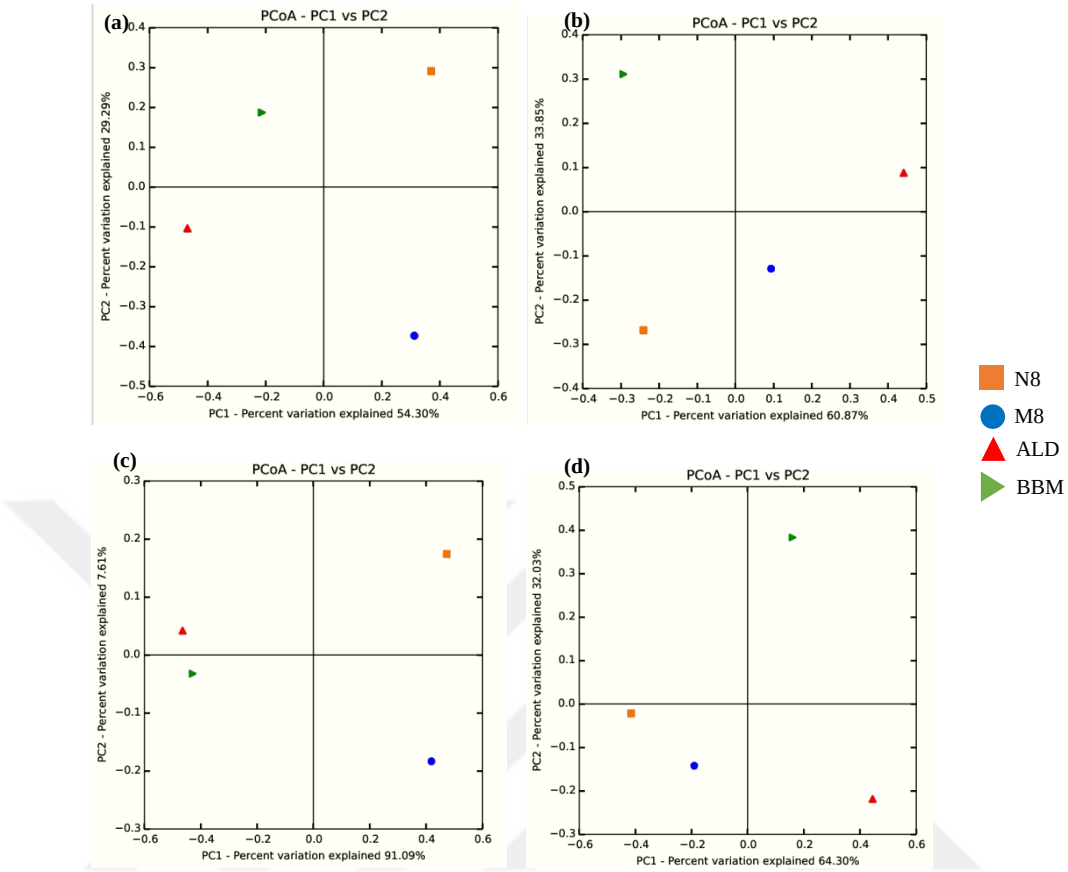


Figure A. 4 : Principal-coordinate analysis (PCoA) performed for (a) 16S rRNA, (b) 18S rRNA, (c) 23S rRNA and (d) tufA marker regions based on Bray-curtis distance matrix.

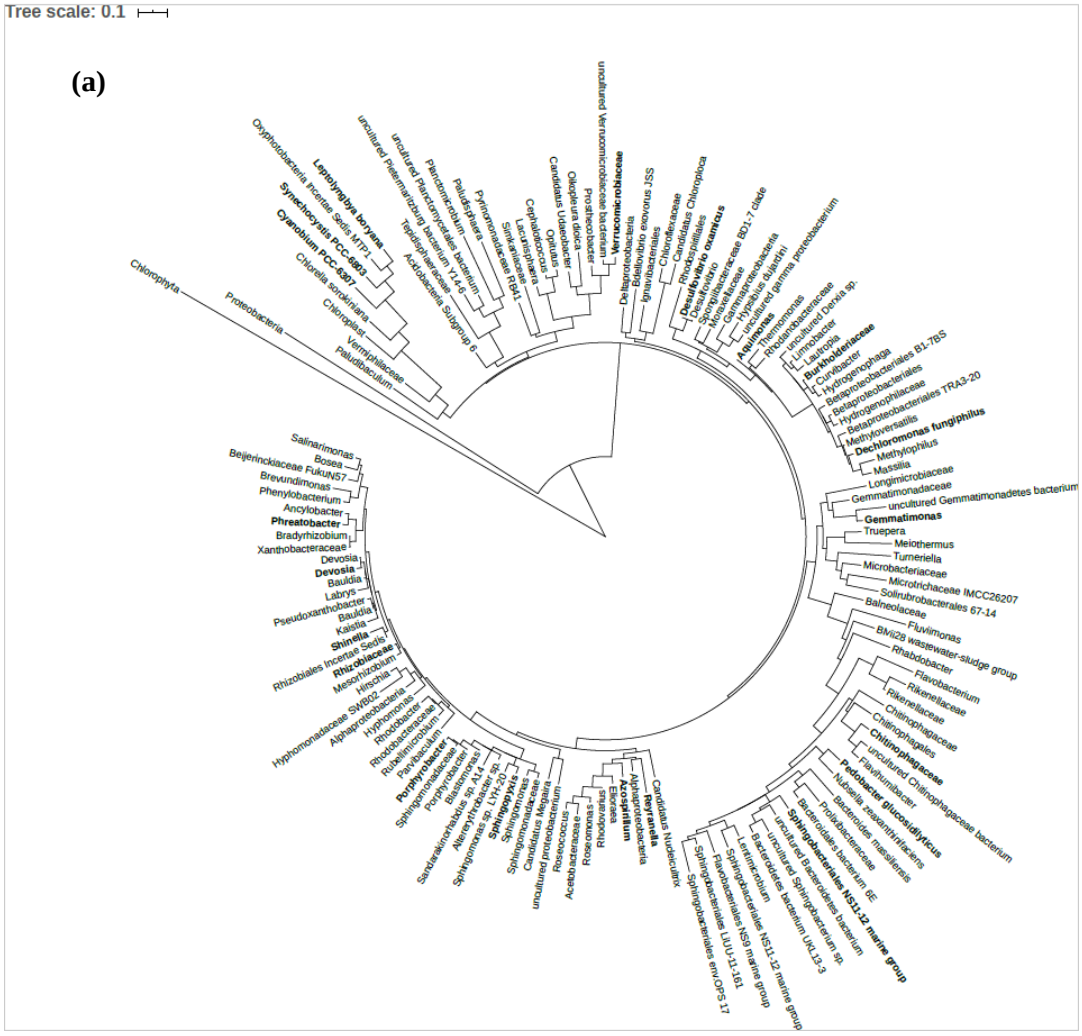


Figure A. 5 : Phylogenetic trees showing the relationship of (a) 16S rRNA, (b) 18S RNA, (c) 23S rRNA and (d) tufA gene sequences. All the phylogenetic trees were constructed with a minimum total feature frequency of 100. Phylogenetic analyses were performed with maximum-likelihood method and visualized using iTOL.

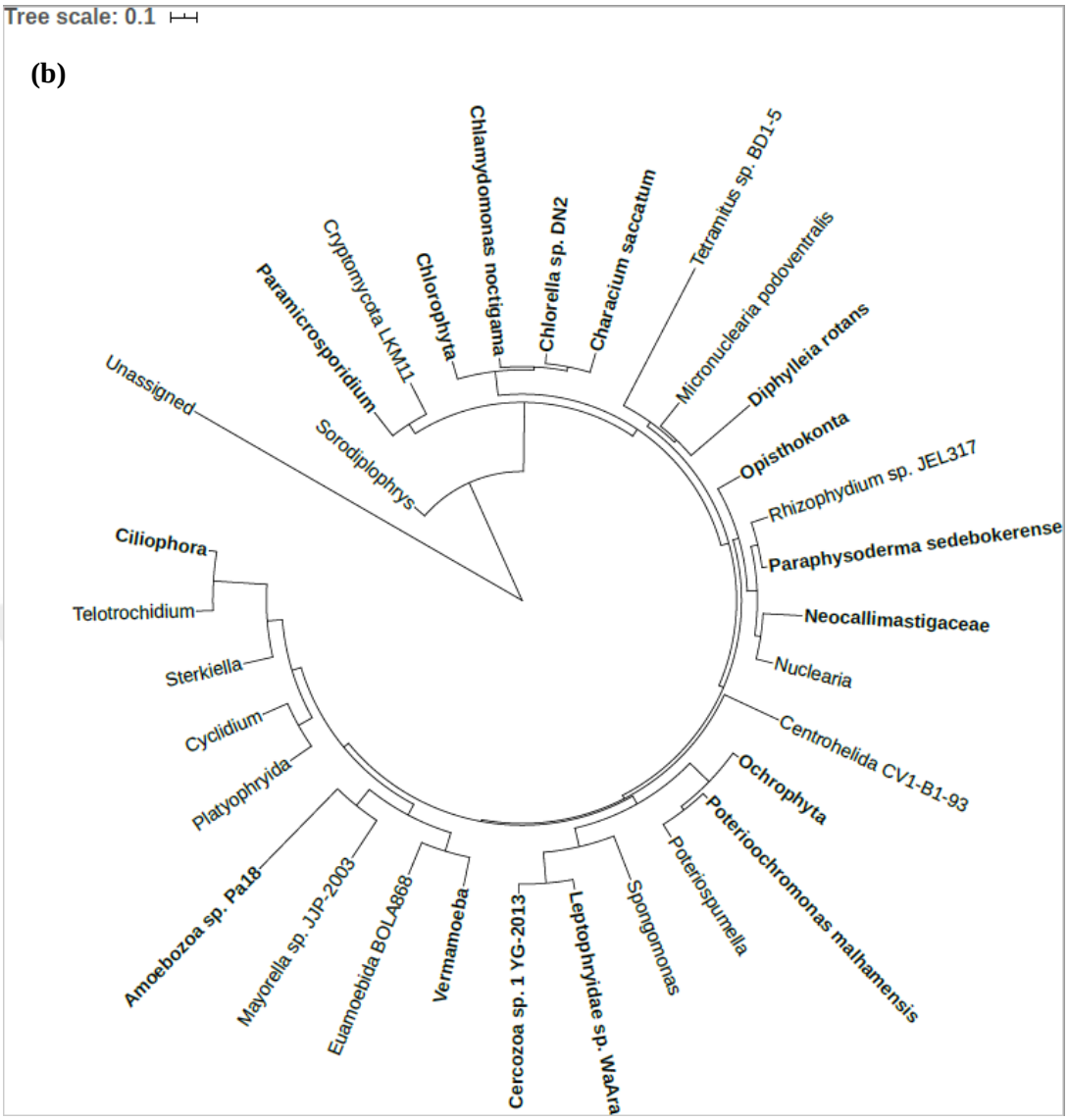


Figure A. 6 (continued) : Phylogenetic trees showing the relationship of (a) 16S rRNA, (b) 18S RNA, (c) 23S rRNA and (d) tufA gene sequences. All the phylogenetic trees were constructed with a minimum total feature frequency of 100. Phylogenetic analyses were performed with maximum-likelihood method and visualized using iTOL.

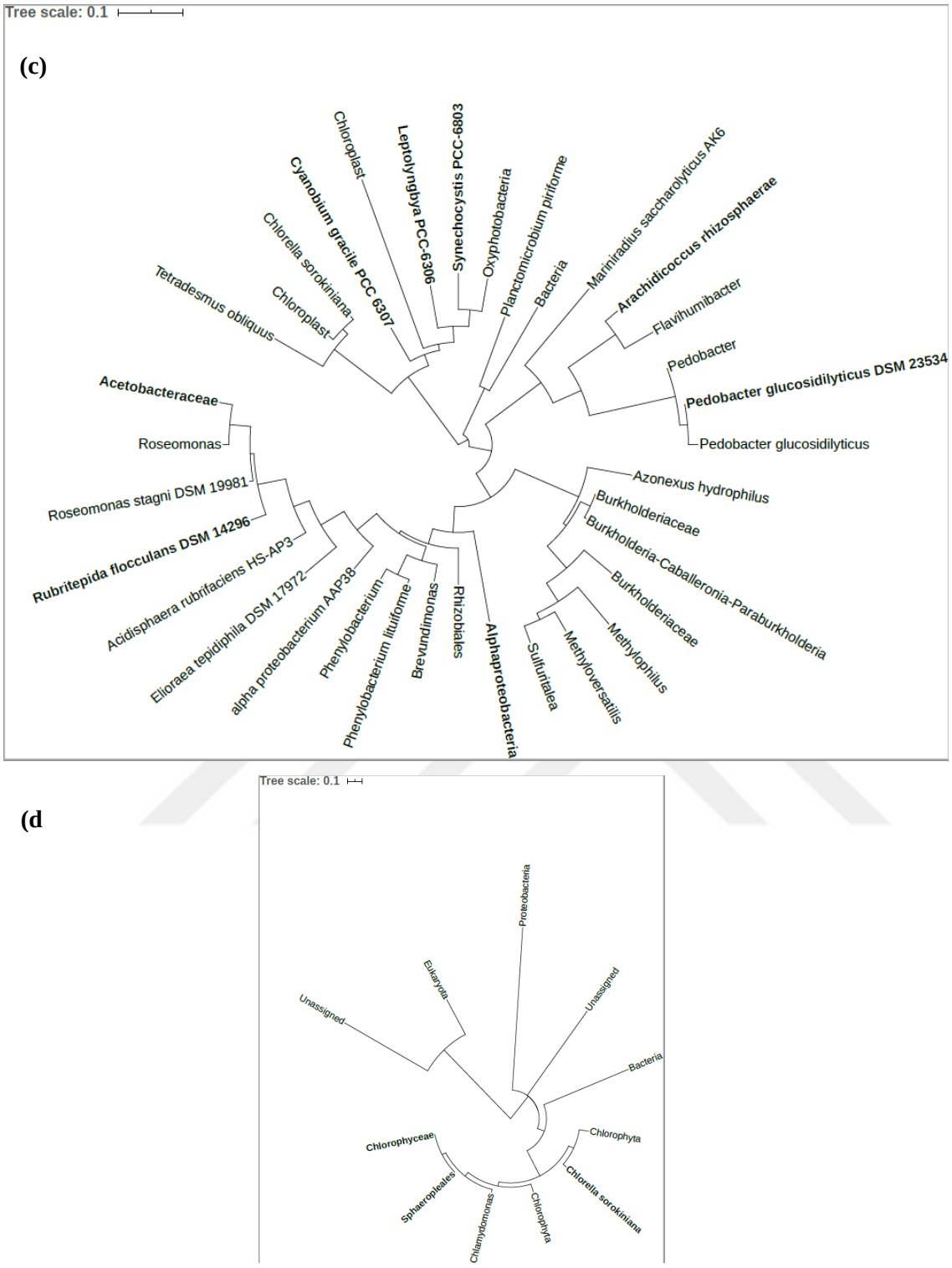


Figure A. 7 (continued) : Phylogenetic trees showing the relationship of (a) 16S rRNA, (b) 18S RNA, (c) 23S rRNA and (d) tufA gene sequences. All the phylogenetic trees were constructed with a minimum total feature frequency of 100. Phylogenetic analyses were performed with maximum-likelihood method and visualized using iTOL.

Table A. 1: Sequence information from QIIME 2 processing of NGS amplicon reads.

	Samples	Number of Reads	Number of sequences after denoising with Dada2	Number of observed ASVs
16S rRNA	ALD	59141	52642	670
	M8	57872	49357	359
	N8	102417	81367	405
	BBM	63624	55567	396
18S rRNA	ALD	70264	48825	102
	M8	132970	79867	88
	N8	110122	47004	68
	BBM	68900	46094	64
23S rRNA	ALD	74140	52553	91
	M8	94088	73623	72
	N8	99084	80347	60
	BBM	96752	70533	48
tufA	ALD	14610	11913	53
	M8	10753	7859	41
	N8	9716	6757	40
	BBM	11132	8725	58

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- Hande Ermiş has involved in many research projects in the field of microalgae.
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PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Ermis, H.,** & Altinbas, M. (2020). Effect of salinity on mixed microalgae grown in anaerobic liquid digestate. Water and Environment Journal.
- **Hande, E.,** Unzile, G. G., Tunahan, C., & Mahmut, A. (2020). Effect of iron and magnesium addition on population dynamics and high value product of microalgae grown in anaerobic liquid digestate. Scientific Reports (Nature Publisher Group), 10(1).
- **Ermis, H.,** & Altinbas, M. (2019). Determination of biokinetic coefficients for nutrient removal from anaerobic liquid digestate by mixed microalgae. Journal of Applied Phycology, 31(3), 1773-1781.

- Microalgae Growth in Anaerobic Digestate For High-Value Product Recovery, Conference Paper, page. 73., ISBN: 978-605-4697-17-5.
- Detection of the dilution factors for anaerobic digestate to cultivate mixed microalgae and the particulate matters effect on cultivation, ICOCEE E-Book, page:2005.

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

- Atıksulardaki Patojen Mikroorganizmaların Toprak ile Giderimi, Book Chapter, sf. 486-513, E-ISBN:978-605-4303-80-9
- Cultivation of Spirulina platensis Using Anaerobic Digestion Effluent As a Nutrient Source For Biomass Production, ICOCEE E-Book, page:2519.