

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**EFFECTS OF NUTRIENTS ON MICROALGAE GROWTH IN ANAEROBIC
DIGESTATE**



M.Sc. THESIS

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Department of Environmental Engineering

Environmental Biotechnology Programme

MAY, 2018

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**MİNERALLERİN ANAEROBİK ÇIKIŞ SUYUNDA ÇOĞALTILAN
MİKROALGLERE ETKİSİ**

YÜKSEK LİSANS TEZİ

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To my family



FOREWORD

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ABBREVIATIONS

COD	: Chemical Oxygen Demand
Ch	: Chlorophyll
CH₄	: Methane
C:N	: Carbon to Nitrogen Ratio
d	: day
DWW	: Domestic Wastewater
EDTA	: Ethylenediaminetetraacetic acid
IEA	: International Energy Agency
GHG	: Green House Gases
HEPES	: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
H₂S	: Hydrogen Sulphate
Mtoe	: Million Tonnes of Oil Equivalent
NH₄-N	: Ammonium as Nitrogen
NH₃-N	: Ammonia as Nitrogen
PO₄-P	: Orthophosphate as phosphorus
OFMSW	: Organic Fraction of Municipal Solid Waste
OD	: Optical Density
WWT	: Wastewater Treatment
WWTP	: Wastewater Treatment Plant
VS	: Volatile Solids
VFAs	: Volatile Fatty Acids
VSS	: Volatile Suspended Solids
VSS_{algae}	: Biomass of the microalgae
TAN	: Total Ammonia Nitrogen
Td	: Generation time or doubling time
TKN	: Total Khenjdalh Nitrogen
TSS	: Total Suspended Solids



SYMBOLS

Al	: Aluminium
Ag	: Silver
B	: Boron
C	: Carbon
Ca	: Calcium
Cd	: Cadmium
Cl	: Chlorine
Cr	: Chromium
Co	: Cobalt
Cu	: Copper
Fe	: Iron
Fe⁺²	: Ferrous Iron
Fe⁺³	: Ferric Iron
H	: Hydrogen
N	: Nitrogen
Na	: Sodium
S	: Sulphur
Si	: Silicium
Sr	: Strontium
Mg	: Magnesium
P	: Phosphorus
Pb	: Lead
Ni	: Nickel
Mn	: Manganese
Mo	: Molybdenum
μ	: Specific Growth Rate
v	: Volume
Zn	: Zinc



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EFFECTS OF NUTRIENTS ON MICROALGAE GROWTH IN ANAEROBIC DIGESTION

SUMMARY

Today it is known that the world population has passed seven billion, and by 2050 it is predicted that the population will be 9.72 billion. In the world, 80% of energy demand are still met by fossil fuels such as oil, coal and natural gas. In addition, 85% of crude oil is used for transportation, and 10% is used for chemical industries. This has led to worldwide interest in biomass production related to biofuel concept.

In present, various products are being produced in different industrial areas from the biomass obtained from various organisms. Bioethanol, biomethane, biohydrogen and biodiesel are obtained from the biomass of the organisms. For biofuels, it is known that plants such as corn, soybean and sugar cane are used. The most important advantage of biofuel production from biomass is the reduction of CO₂ emissions in the air. By using solar energy, CO₂ is converted into organic mass, and biofuel is obtained from this organic biomass. However, the need for a large area for the cultivation of crops, the reduction of agricultural land, and the consumption of too much water are among the reasons why biomass is not kept warm. Algae, who needs less space and water, is now being worked on biofuel studies and many different studies. Especially the ability of microalgae to adapt to different environments has made it possible to obtain various products, including biofuels.

The most important problem in obtaining products from biomass of microalgae is the low microalgal biomass productivity. The microalgal biomass is not sufficient, especially for biofuel such as biodiesel. There are many factors that limit biomass formation. For photosynthetic microalgae, light, temperature, amount of CO₂ and nutrients are main limiting factors. In present, microalgae can be optimized by changing light intensity, temperature, and CO₂ in order to increase its biomass. However, the issue of how much nutrient required or nutrient optimization are still debated. Either the amount of some nutrients may be useful for some microalgae or may be inhibitory or toxic for another microalgae. In addition, nutrient needs may be variable depending on the microalgae species.

It is known that microalgae is cultivated in the anaerobic digestate. The obtained biomass is also used as co-digestion or directly for methane production. The biofuel obtained from microalgae biomass are known to be suitable for biodiesel production, but the obtained biomass is not sufficient in the industrial view. There are many factors affecting microalgae biomass concentration like the dark color and particulate matter of the digestate that limit the penetration of light. Although this problem can be overcome by diluting the digestate or decreasing the reactor volume. The another approach is thought that this problem can be solved by adding nutrients. Some studies made for this purpose and showed that the increase of biomass in wastewater by adding nutrients such as Mg and Fe. The use of wastewater is not only important for the treatment, it is also important factor that reduces water footprint and nutrient requirement to produce microalgae. Because one of the major problems in the world

is the need for clean water. It's now known that using drinking water resources such as lake water for obtaining microbial biomass for industrial purposes is not sustainable environmental way. In addition, drinking water resources is not nutrient enough to produce microalgae. Therefore, microalgae produce in seawater seems to be more attractive because sea water contains nutrients such as Mg and K. However, possible negative impacts on marine ecosystems are still a big question when large facilities are constructed to produce microalgae in the seabed. Considering the flexible adaptation capabilities of microalgae, the use of wastewater in terms of nutrient requirements is now considered as sustainable environmental approach.

In this study, the aim is to increase biomass of the freshwater mixed microalgae cultured in digestate by adding various nutrients referred to BG11 media. For this aim, 600 ml batch cultures were prepared contained digestate with 1:10 dilution ratio. HEPES buffer was used and pH maintained between 7 and 7.5. Different nutrients were added to the each culture and microalgae growth was observed by VSS, chlorophyll measurement and cell counting techniques. Specific growth rate and doubling time were determined based on cell counting data. Additionally, their $\text{NH}_3\text{-N}$, organic C, TKN and $\text{PO}_4^{3-}\text{-P}$ removal efficiencies were also determined by using standart methods, whether there was an effect of removal efficiency or not.

During the experiments, the growth rate and doubling time of the microalgae cultivated in the digestate were lower than BG11 media. Fe source, Mg source and EDTA were added to solutions separately in terms of observing their effect on growth. Fe source was "Ferric Ammonium Citrate" and Mg source was MgSO_4 . When Fe source was increased to 10 times as 3.0mM referred to BG11 media, inhibition was observed in growth. Mg was expected to support growth well, but Mg solutions' results showed lower values compared to EDTA. Among the Mg solutions, only 3.0 mM Mg increased the growth rate and doubling time like $0.1585 \mu^{-1}$ and 4.3 day respectively. When Mg and EDTA added to solutions in different concentrations, Mg 0.6 +EDTA 0.024 mM had the highest growth rate and doubling time like $0.1668 \mu^{-1}$ and 4.1.day respectively.

Chlorella sp. and *Scenedesmus sp.* were observed as dominant species. Rather than microalgae, protozoa with silia or flagella were observed in the digestate media. COD removals were found very low compared to ammonical nitrogen and phosphorus removals. Based on low COD removals, it was considered that there was probably no mixotrophic growth.

The ammonical nitrogen removal rate and the theoretical biomass production were determined for each solution in terms of TKN values via the mass balance calculations. The theoretical biomass production were found higher then experimental biomass production in all solutions.

EDTA was known as a chelator in the media for microalgae and was intended to ensure metal ions remain dissolved in the media to be taken into by microalgae. Among the nutrients, digestate solution with 0,024mM EDTA had the highest biomass concentration with the value 1410 mg /l $\text{VSS}_{\text{algae}}$ with $0.1700 \mu(\text{d}^{-1})$ and 4 days doubling time. It had also the highest net VSS value as 1000 mg/L. And the ammonical nitrogen removal rate and nitrogen removal efficiency showed in EDTA 0.024 mM as 11.4 mg/l/d and 77% respectively.





MİNERALLERİN ANAEROBİK ÇIKIŞ ATIK SUYUNDA ÇOĞALTILAN MİKROALGLERE ETKİSİ

ÖZET

Günümüzde dünya nüfusunun yedi milyarı geçtiği bilinmekte ve 2050 yılına kadar nüfusun 9.72 milyar olacağı öngörülmektedir. Dünyada enerji ihtiyacının %80'i hala petrol, kömür ve doğalgaz gibi fosil yakıtlardan karşılanmaktadır. 2050 yılı enerji tüketimi için öngörülen en iyi senaryolara göre %1,5 ekonomik büyüme ile 9 milyarlık dünya nüfusu fosil yakıtları 2110 yılına kadar kullanacaktır. En kötü senaryoya göre ise %3 ekonomik büyüme ve %100 fosil yakıt tüketimi gerçekleşirse, 2047 yılında bütün fosil yakıtların tükeneyeceğidir.

Yine yapılan araştırmalar ham petrolün %85'inin ulaşım için kullanıldığının %10 luk kısmının kimyasal endüstrilerde kullanıldığını göstermektedir. Bu durum biyokütleden biyoyakıt elde etme çalışmalarının dünyada ilgi çekmesine sebebiyet vermiştir.

Biyokütle canlıların belli bir alanda ve hacimde olan miktarı ya da ağırlığıdır ve g/m^3 ya da g/L olarak ifade edilir. Çeşitli canlıların biyokütlesinden günümüzde farklı endüstri alanlarında bir çok ürün elde edilmektedir. Yine biyokütleden biyoyakıt olarak biyoetanol, biyometan, biyohidrojen ve biyodizel elde edilmektedir. Biyoyakıt için mısır, soya fasulyesi ve şeker kamışı gibi bitkilerin kullandığı bilinmektedir. Biyokütleden biyoyakıt üretiminde en önemli avantaj havadaki CO_2 emisyonunu azaltmasıdır. Güneş enerjisi ile CO_2 fiksile edilerek organik kütleye çevrilmekte ve bu organik kütleden biyoyakıt elde edilmektedir. Ancak dezavantajları fazladır. Bitkilerin yetiştirilmesi için geniş alana ihtiyaç duyulması, tarım alanlarının daraltma etkisi ve fazla su tüketimin olması biyokütleden biyoyakıt elde edilmemesine sıcak bakılmamasının nedenleridir. Bitkilere göre daha az alan ve su ihtiyacı olan algler, günümüzde biyoyakıt çalışmaları için uygun görülmektedir. Özellikle mikroalglerin farklı ortamlara adaptasyon gösterebilmesi mikroalglerden biyoyakıt da dahil çeşitli ürünlerin elde edilmesini sağlamıştır.

Günümüzde mikroalglerden çeşitli ürünler elde etme konusunda en önemli sorun elde edilen biyokütlenin az olmasıdır. Özellikle biyoyakıt eldesi konusunda mikroalglerin ürettiği biyokütle endüstriyel anlamda yeterli değildir. Biyokütle sorunun aşılması için farklı metabolik yolları olan mikroalgler kullanılmış ancak her birinin kendine özgü avantaj ve dezavantajları ortaya çıkmıştır. Mikroalgler metabolizmalarına göre heterotrof, mikсотrof, fotoheterotrof ve fotoototrof olarak dört gruba ayrılır. Bu metabolik ayrımlar bazı mikroalg türleri için çok keskin olmayıp değişen ortam koşullarına göre metabolik yollarını değiştirebilmektedirler. Mesela *C.vulgaris* türü mikroalg değişen ortam koşullarına göre hem fotoototrof hem de mikсотrof büyüyebilir. Heterotrof mikroalglerin sayısı azdır ve çevredeki organik karbonu alarak büyüyebilmektedirler. Ancak büyümeleri için glikozu organik karbon kaynağı seçmeleri, steril ortamda çoğalma ve O_2 ihtiyaçları heterotrof mikroalglerin

biyokütlesinden çeşitli ürünlerin eldesi konusunda büyük dezavantajlardır. Mikrotrof büyümede mikroalgler ortamda ışık ve CO₂ olduğunda fotoototrof gibi büyür, ortamda ışık olmayınca organik karbon olarak glikoz ve asetat gibi maddelerin bulunması zorunluluğu, miksotrof mikroalglerin biyokütlesinden çeşitli ürünlerin eldesi konusunda büyük dezavantajdır. Miksotrofik büyüme genelde fotoheteretrof büyüme ile karıştırılır. Aralarındaki önemli fark, fotoheteretroflar ortamdan organik karbonu almak için ışık enerjisine ihtiyaç duyar; ancak, miksotroflar ortamdan organik karbonu karanlıkta alır, ışık enerjisine ihtiyaç duymaz. Fotosentetik mikroalglerin büyüme hızları ve biyokütleri heterotroflardan ve miksotroflardan az olmasına rağmen biyokütle çalışmalarında yine fotosentetik mikroalgler tercih edilirler. Büyük ölçekli çalışmalar için maliyetinin ucuz olması, esnek adaptasyon yetenekleri ve CO₂ emisyonunu azaltarak bunu organik biyokütleyle çevirmesi fotosentetik mikroalglerin biyokütle eldesinde tercih edilmesinin önemli sebepleridir.

Fotosentetik mikroalgler için fotosenteze bağlı olarak ışık, sıcaklık, CO₂ miktarı ve mineraller belirli kısıtlayıcı faktörlerdir. Günümüz çalışmalarında mikroalglerin biyokütlesi optimize edilmiş ışık, sıcaklık ve CO₂ ile belli miktarlarında artırılabilir. Ancak mineral ihtiyacı ve minerallerin ne kadar gerekli olduğu konusu hala tartışmalıdır. Kimi algler için yararlı olan elementin miktarı başka bir mikroalg için inhibe edici olabilir ya da yetersiz olabilir. Yine mikroalgin türüne göre ihtiyacı olan mineral ihtiyacı değişken olabilir.

Günümüzde mikroalglerin anaerobik çıkış suyu gibi atıksularda çoğalabildikleri bilinmektedir. Mikroalglerin esnek adaptasyon yetenekleri düşünüldüğünde mineral ihtiyacı bakımından göl suyu ya da içme suyu yerine atıksu kullanılması bugün sürdürülebilir çevreci bir yaklaşım olarak düşünülmektedir. Günümüzde endüstriyel amaçlar için mikroalgler içme suyu kaynaklarını kullanarak çoğaltılmakta ve bu durum sürdürülebilir çevreci bir yaklaşım değildir. Ayrıca içme suyu kaynaklarında mikroalg çoğaltmak için yeterli mineral yoktur ve mineral eklenmesi biyokütle artırılması bakımından sık sık uygulanan bir yöntemdir. Bu sebeple bazı çalışmalarda deniz suyunda mikroalg çoğaltılması deniz suyunun Mg ve K gibi mineralleri içermesinden dolayı daha cazip görünmektedir. Ancak deniz suyunda mikroalg çoğaltmak için büyük tesisler inşaa edildiğinde özellikle deniz ekosistemine vereceği olumsuz etkiler de hala bir soru işaretidir. Biyokütlenin anaerobik çıkış suyu gibi çeşitli atık sularda oluşturulması bugün mikroalglerin su ve mineral ihtiyacı düşünüldüğünde sürdürülebilir çevreci bir yaklaşım olarak düşünülmektedir. Ancak her durum gibi, atık suda mikroalg çoğaltılmasının da kendince avantajlar ve dezavantajları vardır. Anaerobik çıkış suyu düşünüldüğünde, anaerobik çıkış suyunun koyu rengi ve partikül yapısı fotosentez için gerekli olan ışık geçirimini olumsuz yönde etkilemektedir. Bu ışık problemi anaerobik çıkış suyunu seyreltme ya da reaktör hacmini düşürmeyle aşılabılır. Bu sorunun çözümü için başka bir yaklaşım da atık suya fazladan mineral eklenmesidir. Bazı çalışmalar atık suya Mg ve Fe gibi mineraller ek olarak eklendiğinde mikroalglerinin büyümesini desteklediğini ve biyokütle miktarını artırdığını göstermiştir.

Bu çalışmada, BG11 ortamında büyüyen karışık mikroalg kültürü kullanılmıştır. BG11 ortamındaki mineraller baz alınarak, anaerobik çıkış suyunda çoğalan mikroalg ortamına çeşitli mineraller eklenerek mikroalglerin biyokütle miktarını artırması amaçlanmıştır. Bu amaç doğrultusunda reaktör hacmi 600 ml olan ve 1:10 oranında seyreltilmiş anaerobik çıkış suyu bulunan ortamlara mikroalgler ekilmiştir. Her bir ortama farklı mineraller belli konsantrasyonlarda eklenerek mikroalglerin büyümleri

uçucu askıda katı madde miktarı, klorofil ölçümü ve hücre sayımı yöntemleri ile takip edilmiştir. Hücre sayımına göre spesifik büyüme hızı ve ikilenme süreleri hesaplanmıştır. Yine deney boyunca pH'ı 7-7.5 arasında sabitlemek için HEPES tamponu-kullanmıştır Eklenen minerallerin amonyak azotu, ortofosfat, organik azot ve organik karbon giderimlerine etkisi olup olmadığı da gözlemlenmiştir. Kontrol grubu olarak mikroalg içeren ve 1:10 oranında seyreltilmiş anaerobik çıkış suyu kullanılmış ve içine hiç bir mineral eklenmemiştir. Deneyler büyüme grafiğinde denge fazına gelene kadar sürmüş, buharlaşan su miktarı numune almadan önce çeşme suyu eklenerek giderilmiştir.

Çalışma boyunca BG11 ortamında çoğalan mikroalglerin anaerobik çıkış suyunda büyüyen mikroaglre göre daha hızlı büyüme hızı ve ikilenme süresi olduğu gözlenmiştir. Fe kaynağı, Mg kaynağı ve EDTA hazırlanan ortamlara ayrı ayrı konulmuştur. Fe kaynağı olarak BG11 ortamındaki ferrik amonyum sitrat kullanılmıştır ve hazırlanan ortamlara 0.3, 0.6, 1.2, 2.4 ve 3.0 mM olacak şekilde eklenmiştir. BG11'deki konsantrasyonundan 10 kat fazla Fe kaynağı ortama 3.0 mM olarak eklendiğinde mikroalglerin büyümesinde inhibisyon yarattığı görülmüştür.

Magnezyum kaynağı olarak BG11 ortamındaki $MgSO_4$ kullanılmıştır ve farklı konsantrasyonlarda 0.3, 0.6, 1.2, 2.4 ve 3.0 mM olacak şekilde hazırlanan ortamlara eklenmiştir. Ancak büyümede ve arıtımda beklenen etki EDTA eklenildiği kadar olmamıştır. Magnezyum solüsyonlarındaki en iyi değer $0.1585 \mu^{-1}$ büyüme hızı ve 4.3 gün ikilenme süresi ile 3.0 mM Mg kaynağında görülmüştür. Mg, EDTA ile beraber farklı konsantrasyonlarda ortamlara eklendiğinde, $0.1668 \mu^{-1}$ büyüme hızı ve 4.1 gün ikilenme süresi ile Mg 0.6 +EDTA 0.024 mM en iyi sonucu göstermiştir.

Deney boyunca *Chlorella* ve *Scenedesmus* türleri baskın tür olarak gözlemlenmiştir. Hazırlanan anaerobik çıkış suyu ortamlarında silli ve flagellalı protozoalara rastlanmıştır. Kimyasal oksijen ihtiyacı giderimleri, amonyak azotu ve ortofosfat giderimine göre çok düşük çıkmıştır. Kimyasal oksijen ihtiyacındaki düşük giderimler değerlendirildiğinde muhtemelen mikсотrof büyüme olmadığı düşünülmüştür.

Kütle dengesi formülü kullanılarak, TKN giderimlerine göre her bir hazırlanan solüsyonun amonyak azotu giderimi hızı ve teorik biyokütle oluşumu hesaplanmış ve deneysel sonuçlarla karşılaştırılmıştır. Hazırlanan her solüsyonda teorik biyokütle miktarı deneysel olarak elde edilen biyokütleden fazla çıkmıştır.

Şelat olarak tanımlanan EDTA, büyüme ortamındaki minerallerin çözünmüş halde kalmasını sağlayan madde olarak tanımlanır. Minerallerin çözünmüş halde ortamda kalmasını sağladığı için ortamdaki mikroorganizmaların büyümesini desteklediği belirtilir. Deneylerde EDTA'nın büyümeyi desteklediği gözlemlenmiş ve mikroalg ortamlarına 0.0024, 0.0048, 0.0144 ve 0.024 mM konsantrasyonlarda eklenerek büyüme izlenmiştir. Deneylerin sonucunda büyümeyi ve biyokütle konsantrasyonu artıran 0.0144 mM ve 0.024mM konsantrasyonda EDTA olmuştur. Amonyak azotu giderimi hızı, amonyak azotu giderimi ve ortofosfat giderim yüzdesi de göz önüne alındığında 0.0144 mM EDTA solüsyonuna göre 0.024 mM EDTA tüm hazırlanan solüsyonlar içinde en iyi sonucu göstermiştir. $0.1700 \mu(d^{-1})$ spesifik büyüme hızı ve 4 günlük ikilenme süresi ile 0.024mM EDTA en yüksek biyokütle verimine 1410 mg VSS_{algae}/l olarak ulaşmıştır. Yine net biyokütle miktarlarına bakıldığında 0.024 mM EDTA 1000 mg/L net biyokütle oluşumuyla en yüksek değere sahiptir. En yüksek amonyak azotu giderim hızına 11.4 mg/l/gün olarak 0.024 mM EDTA ile ulaşılmıştır. En yüksek amonyak azotu giderim yüzdesine yine 0.024 mM EDTA'yla %77 oranında ulaşılmıştır.



1. INTRODUCTION – ENERGY DEMAND OF THE WORLD

World energy consumption has duplicated in past 40 years, reaching 8,000 Mtoe, of which 80 % is still covered by fossil fuels that are oil, coal, and natural gas based on data by IEA (Formighieri, 2015). In 2001, energy consumption with 6.1 billion people was 13.5 TW; 86% obtained from fossil fuels. (Tabassum et al, 2017). In 2012, 82% of total primary energy consumption is made up of fossil fuels including coal, oil and natural gas (Correa et al, 2017).

World primary energy consumption is expected to double by 2050 with a 1.6 % annual growth rate (Tabassum et al, 2017). It is estimated that by the year 2050 the world's population will reach 9.72 billion (Tandon and Jin, 2017). Based on scenario considering 9 billion world population with a 3 % economic growth and 100 % fossil fuels use, fuel reserves would be finished by 2047 . Based on the best scenario with considering with a 1.5 % economic growth, fossil fuel reserves would be finished by 2110 (Formighieri, 2015). Almost 85% of the crude oil is used for the transportation and 10% for production of chemicals in industries. This undoubtedly is an area of concern and the reason why production of alternative renewable energy sources attracts worldwide attention (Tandon and Jin, 2017), especially for biomass related to microalgae.

Biomass is defined as total quantity or weight of organisms in a given area or volume and generally expressed as g/m^3 or g/L . In microalgae system, microalgae consume nutrients in wastewater or water and producing their own biomass with photosynthesis. Biomass as a kind of renewable energy, specifically biofuels, has become important related to energy, environment and economics. Microalgae biomass has received much attention for biofuels, obtaining valuable products, wastewater/water treatment lately because of its suitability of biotechnological processes considering a cradle-to-cradle bio-based economy (Puyol et al, 2017). Macroalgae can have higher growth rates and higher rate of CO_2 fixation than land-based energy crops (Tabassum et al, 2017).

1.1 Purpose of Thesis

Purpose of thesis was to determine specific nutrient(s) for mixed microalgal culture cultivated in media containing 10% digestate in order to increase biomass concentration. Anaerobic digestion is commonly used process to obtain biogas (methane) from biodegradable wastes to provide heat, electricity or both. After this process, the effluent is called as “Anaerobic Digestate” or only “Digestate” that is strong wastewater but it also has valuable raw materials like nutrients inside that can be recovered. Generally, the digestate is used as a fertilizer, which is spread to farmland. But its transportation cost is high. On the other hand, using the digestate as fertilizer may also cause secondary microbial pollution because of its pathogens potential. Therefore, to find an environmentally friendly way for the digestate treatment is essential. Microalgae have an ability of growing in anaerobic digestate in a suitable concentration and promising an environmental friendly way for the treatment of anaerobic digestate. During growing process, they take nutrients from digestate and produce their own biomass. This produced biomass can be used as fertilizer, biogas production or acquisition biofuel including biodiesel. Nutrients are very important for all microalgae species’ growth and can be a key for increasing biomass productivity.

In this study, it was purposed to determine the requirement of specific nutrient(s) to increase microalgae biomass cultivated in digestate. Mixed microalgal culture was cultivated in digestate diluted with 1:10 ratio. 1:10 dilution was chosen as suitable concentration based on pervious studies carried out on the same digestate as this study. Variable nutrients were added to anaerobic digestate regarding nutrients as present in BG11 media. Ferric ammonium citrate as Fe source, MgSO_4 as Mg source and EDTA were added to solutions separately in terms of observing their effect on growth. At the end of all cultivations, nutrient removal efficiencies, growth rates, doubling times and biomass concentrations were determined for all.

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1.2 Scope of The Thesis

The scope of the thesis is given below:

- In the second chapter, the updated literature survey related to the differentiation of microalgae and cyanobacteria, the factors affecting microalgae growth, nutrients importance on the microalgae growth and microalgae cultivation in anaerobic digestate effluent was all given in detail. In this chapter, the aim was to show how important of nutrients for each microalgae process especially in nutrients part, microalgae cultivation in wastewaters' part.
- In the third Chapter, materials and methods used in the experimental set-up and in the theoretical approaches were given in detail.
- In the forth Chapter, results and discussions were given in detail.
- In the fifth chapter, the conclusions and recommendation were given.



2. LITERATURE REVIEW

Algae are divided into two groups based on size: macroalgae and microalgae. Algae growing in the form of large, plant-like structures are called 'Macroalgae'. Microalgae are unicellular Eukaryotic organisms, some of them form colonies that are visible to naked eye. To see unicellular forms, microscope is needed. Cyanobacteria, often known as blue-green algae, are oxygenic photosynthetic bacteria which contain blue-green and green pigments. The main differences between cyanobacteria and microalgae is their domain. Cyanobacteria are in Prokaryotes domain; microalgae are in Eukaryotes domain. In general, the term of "Microalgae" refers to both all cyanobacteria and microalgae species (Brasil et al, 2017; Smetana et al, 2017).

The biomass of microalgae has lately received much attention for biofuels, obtaining valuable products, wastewater treatment because its suitability of biotechnological processes (Puyol et al, 2017). In present, there have been wide applications of wastewater to microalgae cultivation, including municipal wastewater, domestic wastewater, anaerobic Digestion effluent of animal manures (Zhang et al, 2017) and leachate (Garcia et al, 2017) .

Most of microalgae are photoautotrophs. But some microalgae species can change their metabolic pathway into heterotrophic or mixotrophic. For instance, *C. vulgaris* that is photoautotrophic microalgae, can change its metabolic pathway into heterotrophic or mixotrophic based on the media content during cultivation (Zhang et al, 2014; Kandimalla et al, 2016). Based on their metabolic pathway, there are four major types of cultivation conditions for algae: photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic (Zhang, 2013).

Autotrophic metabolism or photoautotrophic metabolism refers to photosynthetic microalgae. Heterotrophic metabolism refers to the acquisition of energy and carbon from organic compounds available from the environment. There are a few microalgae species that are strict heterotroph (Lowrey et al, 2015).

Mixotrophy is a combination of autotrophy and heterotrophy. When a mixotrophic microalgae is used, it will do photosynthesis in light period and use CO₂ ; it will use organic carbon in the dark period and act like heterotrophic organism (Lowrey et al, 2015; Dickinson et al, 2017). Mixotrophy is generally confused with photoheterotrophy and this is normal because they need similar conditions for cultivation. The main difference between mixotrophy and photoheterotrophy is that photoheterotrophy requires light as the energy source to take organic carbons, while mixotrophy take organic compounds without needing light energy (Wang et al, 2014).

Phototrophic microalgae typically have lower lipid productivity and lower growth rate than heterotrophs. Mixotrophic microalgae have the advantage of being able to use multiple food sources, but may require complex cultivation equipment (Dickinson et al, 2017). At this point, the question is normally asked why phototrophic pathway is preferred in biotechnological algal applications. Briefly, using phototrophic microalgae is provide an environmentally friendly way in many cases. Heterotrophic cultivation is not feasible for large scale production (Lowrey et al, 2015; Yu et al, 2017). Potential disadvantages of heterotrophic cultivation of microalgae are to use mainly glucose as carbon source (Wang et al, 2014; Lowrey et al, 2015); the need for sterile media (Lowrey et al, 2015; Yu et al, 2017) and additional O₂; the production of carbon dioxide affecting pH (Lowrey et al, 2015). Mixotrophic and photoheterotrophic cultivation are rare and their operational cost is high compared to photoautotrophs (Yu et al, 2017). For mixotrophic cultivation, organic substances like glucose and acetate should be used to support their growth (Wang et al, 2014). Photosynthetic microalgae can be cultivated in variaty wastewater. They provide to recover nutrients from wastewater and decrease CO₂ level considering green house effect. The biomass obtained can be used for biodiesel production or many applications. Hovewer, understanding metabolic pathways for microalgae is very important to optimize lipid and biomass productivity (Wang et al, 2014; Lowrey et al, 2015; Kandimalla et al, 2016). Nutrients, temperature, light are important factors affecting photosynthetic growth. Therefore, understanding photosynthesis is necessary for using photosynthetic microalgae.

2.1 Photosynthesis

Photosynthesis is the conversion of light energy to chemical energy. Inorganic compounds are converted into organic compounds. Light, chlorophyll, nutrients, CO₂ and H₂O are reactants; organic compounds and O₂ are products in photosynthesis (Figure 2.1). Organisms making photosynthesis are called phototrophs, their sole carbon source is CO₂. Energy from light is used in the reduction of CO₂ to organic compounds (photoautotrophy). However, some phototrophs use organic C as their C source; this lifestyle is called photoheterotrophy.

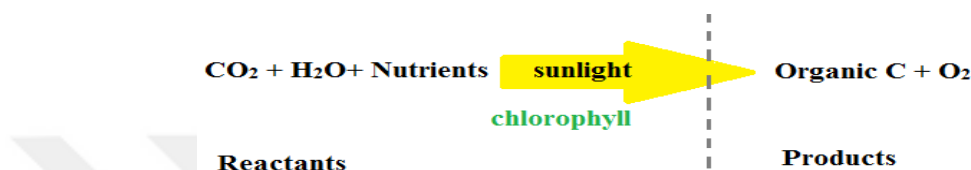


Figure 2.1 : Photosynthesis equation by showing its reactants and products.

Chlorophyll absorbs light energy and starts photosynthesis. It is found in higher plants, most algae, and cyanobacteria. Chlorophyll a is green in color because it absorbs red and blue light preferentially and transmits green light. All oxygenic phototrophs contain chlorophyll; but anoxygenic phototrophs contain bacteriochlorophyll. The main differences between chlorophyll and bacteriophyll is about different wavelengths of light absorption (Figure 2.2). Algae species do not have bacteriophylls and have different chlorophyll types and different pigments. The absorption spectrum of cells containing chlorophyll shows strong absorption of red light (maximum absorption at a wavelength of 680 nm) and blue light (maximum at 430 nm) (Figure 2.3) (Madigan et al, 2010).

Chlorophyll is inside of the chloroplast in microalgae which is a kind of plastids. Prokaryotes do not contain chloroplasts. Both plants and microalgae have similar structure of chloroplasts. The main difference between two is chloroplasts' shape. Microalgae species have various chloroplast shapes.

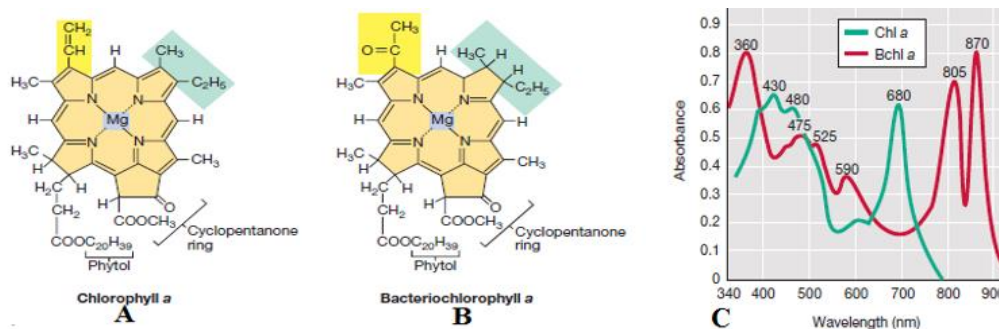


Figure 2.2 : A is chlorophyll structure; B is bacteriophyll structure; C is the differences between chlorophyll and bacteriophyll (Madigan et al, 2010).

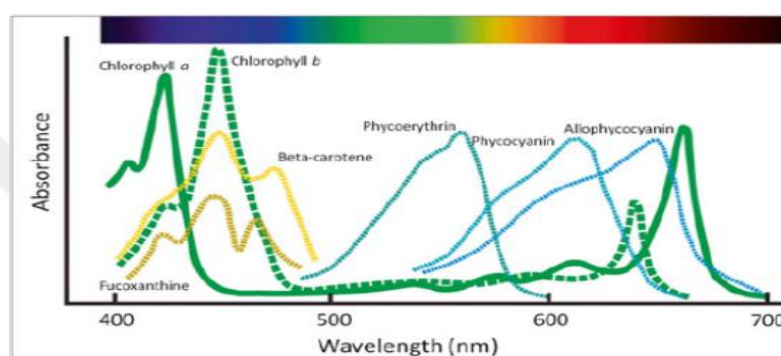


Figure 2.3 : Absorption spectra for various algal pigments (Arora& Sahoo, 2015).

2.2 Limiting Factors Affecting Photosynthesis

To increased Biomass concentration in photosynthetic microalgae is mainly related to photosynthesis efficiency. Simply, the more photosynthesis microalgae does, the more biomass concentration will be produced or obtained. Light, (Iluz and Dubinsky, 2013) , temperature (Huesemann et al, 2016) , pH, nutrients, CO₂/O₂ (Iluz and Abu-Ghosh, 2016) ratio and toxic chemical are abiotic factors; pathogens, predators (zooplankton), competition with bacteria are biotic factors affecting microalgae growth.

2.2.1 Light

Light an important parameter for photosynthetic growth that its absorbtion is affected or limited by many factors like temperature, CO₂ concentration, and nutrient concentration. For microalgae, the water depth and turbidity must be considered for light absorbtion (Formighieri, 2015). The wavelength and intensity of light is very important when photosynthesis efficiency is considered. Phototroph organisms have a

saturation light value during photosynthesis (Figure 2.4) and photosynthesis efficiency is increasing until the reach of light saturation. After saturation, photosynthesis efficiency is become constant (Iluz and Dubinsky, 2013). But, light saturation value can be optimized by changing temperature, CO₂ concentration, and also nutrient concentration.

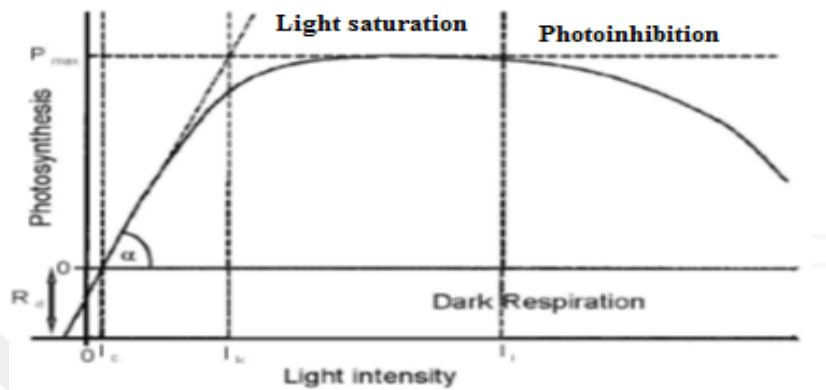


Figure 2.4 : Light response curve of photosynthesis versus light intensity (Iluz and Dubinsky, 2013).

2.2.2 pH

The pH of any media had a big influence on the growth rate (Crofcheck et al, 2012; Silva et al, 2017). Each microalgae have an optimum pH value and pH ranges. High pH values inhibit the uptake of nutrients such as Ca²⁺, phosphates and some nitrogen species. Ca²⁺ ions and phosphates precipitates at high pH level. As it is mentioned in nitrogen section and C source section, NH₃/NH₄⁺ concentration is related to pH. When pH at over 9.3 all NH₄ converts into NH₃ and leaves the system as a gas or diffuses microalgae inside and causes toxicity. To maintain pH at stated ranges, buffer can be used or CO₂ can be giving the system for lab scale studies. To diffuse CO₂ into large scales applications costs too much. HEPES, MES, ADA, PIPES, TRIS can be used (Bartley et al, 2014; Nguyen et al, 2016; Silvia et al, 2017) for pH buffers but these are not recommended to used large scale or pilot scale applications because these buffers might be toxic for microalgae and might inhibit their biomass productivity. They are generally use for pH effect on growth in lab scale experiments (Bartley et al, 2014; Silva et al, 2017).

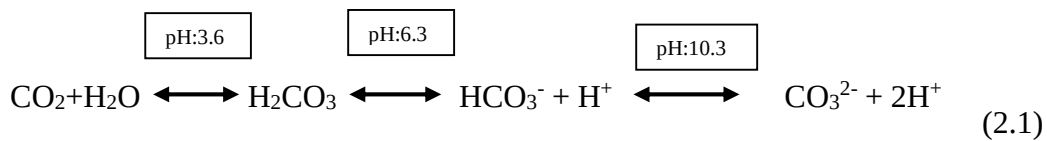
2.2.3 Nutrients

According to Liebig's law of the minimum, when all other factors are in optimum range (e.g., light, temperature), the nutrient available in the smallest quantity will limit the growth of an organism. This law is true when terrestrial plants are considered; but this law makes discussion when microalgae's ability of replacing one nutrient to another one is considered. It is hypothesized that there are synergistic/antagonistic behaviors when one element is present in excess of another for microalgae (Quigg, 2016).

Microalgae requires high concentrations of essential nutrients (C, N, P, S, K, Fe etc.). The general empirical formula for microalgae is described as $C_{106}H_{263}O_{110}N_{16}P$ (Ebeling et al, 2006; Markou et al, 2014; Jazzar et al, 2016). Microalgal biomass has low content of cellulose compared to terrestrial crops and it contains three times higher nutrients than the biomass of terrestrial plants. As a result, the nutrient demand for microalgal biomass production is much higher than agricultural crops (Markou et al, 2014). Although nutrient requirements is considered as an important effect on increasing the productivity of microalgal cultures, little attention has been paid (Ulloa et al, 2012; Napan, et al, 2015; Garcia et al, 2017). Macro nutrients for microalgae are C, N, P, K, Ca, Mg, S, Na, Cl; micro nutrients are Fe, Mn, Zn, Cu, Mo, Ni and Co.

2.2.3.1 CO₂ as carbon source

CO₂ is dissolved in water it reacts with the water molecules (H₂O) and forms a weak acid-base buffer system, having the following equation 2.1 (Markou et al, 2014):



The microalgae thrive between pH 6.5 and 10, the dominant carbon form is bicarbonate HCO_3^- . Lowering pH values, more H_2CO_3 is gradually formed and when pH is 6.3, the concentration of H_2CO_3 is equal to HCO_3^- . At pH lower than 6.3, the H_2CO_3 is predominant. In contrast, increasing pH values more CO_3^{2-} is gradually formed and when pH is 10.3, the concentration of CO_3^{2-} is equal to HCO_3^- . At pH higher than 10.3 the CO_3^{2-} is predominant. (Markou et al, 2014). Many species of microalgae and cyanobacteria can take up both CO₂ and HCO_3^- , some may take up

only CO_2 or HCO_3^- and some extremely alkaliphilic can utilize CO_3^{2-} . CO_2 can be taken up via diffusion or active transport mechanisms. HCO_3^- is taken up only active transport mechanisms with the help of an enzyme called “Carbonic Anhydrase” (CA) that is energy consuming process as it is seen in equation 2.2. This process also produces OH^- , which gradually causes an increase of the pH of the medium, which can reach at 11. Therefore, the control of the pH is a significant issue to maintain microalgal growth. To control the rising pH, a strategy is to acidify the medium, either by applying CO_2 or by using inorganic or organic acids (Markou et al, 2014).



CO_2 is provided by the aeration with air (400 ppm CO_2) and resulting to high energy consumption for its pumping (Markou et al, 2014). Other sources which could be used are biogas derived from the anaerobic digestion process (with a CO_2 content of 20-40%) (Correa et al, 2017; Garcia et al, 2017). Another source for inorganic CO_2 is wastewaters streams that are rich in bicarbonate-carbonate alkalinity.

2.2.3.2 Nitrogen

Nitrogen is the second most abundant element in microalgal biomass. Nitrogen is found nucleic acids (DNA, RNA), proteins and pigments such as chlorophylls. Microalgae have a very high protein content (30-60%) when compared to terrestrial plants and therefore nitrogen requirement is high. (Crofcheck et al, 2012; Markou et al, 2014). Nitrogen species that microalgae utilize are nitrate, NO gas, urea, ammonium and ammonia.

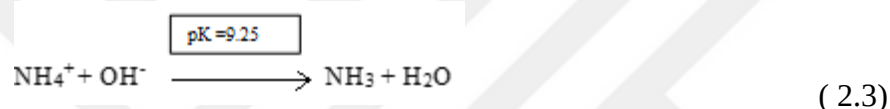
Nitric oxide (NO) is gas form and has a very low solubility in cultivation medium. To improve the solubility of NO , ferrous-complexed EDTA can be added into the cultivation medium (Markou et al, 2014).

Nitrate (NO_3^-) is the most commonly used nitrogen form. The most frequently used nitrate salts are NaNO_3 and, less frequently KNO_3 . NO_3^- is taken up by active mechanisms and therefore consumes energy. The growth is negatively affected when the concentration of nitrate is increased. Possibly, the activity of nitrate reductase is enhanced when NO_3^- concentrations are high and these results in a high intracellular concentration of NO_2^- and NH_4^+ , both of which are toxic to the cells when they are excess (Markou et al, 2014).

Microalgae can utilize nitrogen from organic forms such as urea and some amino acids as organic nitrogen. The most preferred organic nitrogen form is urea for microalgae cultivation. Generally, urea is hydrolyzed to ammonia and carbonic acid which both can be utilized by microalgae (Crofcheck et al, 2012).

2.2.3.3 Ammonia(NH₃) /ammonium (NH₄⁺)

NH₃ is a volatile molecule and gaseous form, but, unlike CO₂, its solubility is very high. When NH₃ is dissolved in water, it reacts with water to form a buffer system of NH₃/NH₄⁺: The equilibrium between the forms of NH₄⁺ and free NH₃ depends mainly on the pH and temperature. At pH values higher than 9.25 at 25°C, the dominant species is the free NH₃ as it is given in equation 2.3 (Markou et al, 2014). However, the degree of the toxicity is species dependent (Markou et al, 2014).



Wastewaters generally contain high NH₃. NH₃ toxicity should be considered when wastewaters will be used as cultivation medium. To avoid the negative effect of free NH₃, a strategy is to regulate the pH values or to dilute the wastewater (Markou et al, 2014; Hajar et al, 2017). If NH₄⁺ is applied as the nitrogen source the pH may drop due to the release of H⁺ during assimilation, while the pH will increase due to the release of OH⁻ when nitrate is applied (Markou et al, 2014).

The simultaneous presence of more than one nitrogen form in the cultivation medium affects the uptake of nitrogen. When NH₃/NH₄⁺ is present, the uptake of nitrate is repressed and microalgae will first complete NH₃/NH₄⁺, and then utilize the other forms. On the other hand, a high nitrate concentration also inhibits NH₃/NH₄⁺ uptake. NH₃/NH₄⁺ also represses urea uptake. The rank of preferred nitrogen utilization is NH₄⁺ > NO > NO₂⁻ > NO₃⁻ > urea (Markou et al, 2014).

2.2.3.4 Phosphorus (P)

Phosphorus is an component of nucleic acids (RNA and DNA), NADPH and NADP in photosynthesis, membrane phospholipids and ATP (Jazzar et al, 2016). In natural environments as well as in wastewaters, phosphorus is present in various forms such as orthophosphate (PO₄³⁻) , polyphosphate and their organic forms. It is well known

that phosphorus is taken up by the cells in the PO_4^{3-} form, however other inorganic and organic phosphorus forms can also be utilized by microalgae.

The uptake rate of phosphorus is affected by the cell condition and by several environmental factors, such as available energy (light), pH, temperature, salinity/ionic strength of the cultivation medium and available ions such as K^+ , Na^+ and Mg^{2+} . (Markou et al, 2014). At increased pH due to photosynthesis and the alkalization of the cultivation medium, Ca^{2+} and Mg^{2+} in the media may precipitate with phosphates and this can reduce the availability of phosphorus especially in wastewaters in which the content of divalent cations is high (Markou et al, 2014).

2.2.3.5 Magnesium (Mg)

Mg is a part of chlorophyll. It is also an activator for several major enzymes. Chlorophyll is a protein and synthesized by the gene in the DNA. The gene in the DNA is not activated to produce chlorophyll when there is no enough light. Mg is the main element for the chlorophyll and it is expected to support growth well.

Magnesium is provided to the cultures mainly as MgSO_4 . The other magnesium salts can be used, such as magnesium-diammonium sulfate $[(\text{NH}_4)_2\text{MgSO}_4]$, magnesium chloride (MgCl_2), magnesium hydrogen phosphate (MgHPO_4), magnesium nitrate $[\text{Mg}(\text{NO}_3)_2]$. Magnesium in aqueous solutions is mainly presented as the Mg^{2+} cation. In this case, alkalinity must be considered related to pH value of the culture. When pH is close to 11, Mg^{2+} may precipitate as magnesium phosphate $\text{Mg}_3(\text{PO}_4)_2$ with phosphate or magnesium hydroxide $\text{Mg}(\text{OH})_2$. These mineral precipitates may induce flocculation of microalgal biomass which is not desirable (Markou et al, 2014).

Additional Mg is required for cultivation microalgae not only for artificial media or freshwater (Ulloa et al, 2012), it is also required when microalgae cultivated in wastewaters like human urine and anaerobic digestion effluents. But the requirement is less in wastewaters compared to artificial media. Bjornsson et al. (2013) cultivated *Scenedesmus* sp. AMDD in the diluted digestate of swine manure and obtained high biomass and nutrient removal efficiency when they added MgSO_4 and MgCl into solution. They declared that Mg itself had high effect on algal biomass productivity. For Bjornsson et al. (2013) study, details is given in “Liquid digestate” section.

2.2.3.6 Micronutrients

Among trace elements, Fe is one of the most essential elements required by microalgae. Fe plays a crucial role in electron transport processes such as respiration and photosynthesis. Fe-containing proteins are also directly involved in nitrate and nitrite reduction, chlorophyll synthesis and the detoxification of O₂ radicals and chlorophyll synthesis (Radzun et al, 2015). In natural environments, and in domestic wastewaters, iron is frequently bio-unavailable and this can limit the growth capability of microalgae and cyanobacteria. Therefore, iron is frequently supplied in cultures as chelated complexes like Fe-EDTA, which render it bio-available. Iron deficient cultures display low growth rates, while high iron concentration in some microalgal species induce lipid synthesis and increasing its content in the biomass. (Markou et al, 2014).

Organisms acquire iron from the environment hardly, because it is found mainly in insoluble Fe³⁺ form. They can take Fe⁺² which is soluble and less because it quickly oxidizes to Fe⁺³ when oxygen is present. As an adaptation example, microorganisms have mechanisms usually enable uptake Fe⁺³, and this mechanism reduces Fe⁺³ to Fe⁺². But extracellular reduction of ferric iron is the limiting step in iron uptake (Bleackley and MacGillivray, 2011).

Manganese (Mn) is deficient as it alters chlorophyll formation. It is a cofactor in several Krebs-cycle enzymes (Quigg, 2016).

Zinc (Zn) is an essential element and cofactor for over 150 enzymes (Radzun et al, 2015). RNA content is low in Zn deficient algal cells. Because of Zn's essential role in DNA metabolism, zinc-limited cells were found to be incapable of completing their mitotic cycle and were unable to undergo cell division (Quigg, 2016). But it can also inhibit the growth of microalgae if present at elevated concentrations or as a result of antagonistic behaviors in the presence of some other micronutrients.

Copper (Cu) is known to play a role in the photosynthetic electron transport chain and the respiratory electron transport chain. It is a component of plastocyanin in "Photosystem I"; a component of cytochrome oxidase that reduces oxygen to water. Cu uptake can be inhibited by pH, ambient light, the presence of other ions like Fe (Quigg, 2016).

Molybdenum (Mo) is essential for the biological assimilation of N like nitrate reductase. Nitrate reductase is used by all organisms in N metabolism for reducing NO_2 to NO_3 , also has a Mo metallo-center. Sulfate has been found to inhibit Mo assimilation by microalgae, making Mo less available in seawater than in freshwater despite its higher concentration (Quigg, 2016).

Cobalt (Co) is known to be required in vitamin B12 (metallo-centre) (Radzun et al, 2015). DNA synthesis and synthesis of thymine, vitamin B12 plays an important role.

2.2.4 EDTA (Ethylenediaminetetraacetic acid)

EDTA is known as a chelator in the media for microalgae and is intended to ensure metal ions remain dissolved in the media to be taken into by microalgae (Zhang et al, 2015).

There are various EDTA species and Fe.EDTA, Na. EDTA.Fe are known to be used in microalgae media. Crofcheck et al. (2012) cultivated *Chlorella vulgaris* and *Scenedesmus acutus* in fresh water based media in which urea was nitrogen source. Urea media was prepared by adding various amounts of EDTA like no EDTA; 0.02 g/L Na.EDTA.Fe; and 0.2 g/L Na.DTPA.Fe(III). At the end of the experiment, they declared that there was no significant difference between both of the EDTA types and the absence of EDTA on the growth productivity. The obtained biomass with Na.EDTA.Fe was 5.64 ± 0.28 mg/L; with Na.DTPA.Fe(III) 6.83 ± 0.23 mg/L and 6.84 ± 0.36 mg/L biomass obtained when there was no media.

Bjornsson et al. (2013) cultivated *Scenedesmus* sp. AMDD in the diluted digestate of swine manure and added trace metals, vitamin stocks and Fe.EDTA. They declared that EDTA increased biomass but not significantly.

2.3 Microalgae Cultivation in Wastewater

Wastewater can be defined as non-drinkable water containing unwanted solids or dissolved particles and organisms inside. Wastewaters are rich in organic and inorganic nutrients, and with their high COD and they pose a threat to environmental safety if untreated (Yu et al, 2017).

To produce valuable products such as commercial products, health products and “Single Cell Protein” as food, wastewater is not preferred in case it contains unwanted

contaminant (Markou et al, 2014; Puyol et al, 2017). When microalgae cultivate in freshwater media to obtain these valuable products, nutrient requirement and water requirement (water footprint) are high to support microalgae growth and these make the cost of microalgae cultivation high compared to using wastewater (Bohutskyi et al, 2015; Guldhe et al, 2017). As for biodiesel production, alternative, low-cost sources of nutrients and water, such as wastewater, could provide advantages for enhancing the long-term sustainability of microalgae cultivation (Markou et al, 2014; Tran et al 2014; Garcia et al, 2017; Yua et al, 2017). Moreover, the GHG like CO₂ decrease when photoautotrophic microalgae are used for cultivation (Figure 2.13) (Garcia et al, 2017; Yu et al, 2017). For instance, Kobayashi et al. (2013) cultivated *Chlorella sp.* in digestate of cattle manure and in BBM media. They found that the total lipid content of *Chlorella sp.* had close similar percentages in both BMM media and in the digestate. This experiment showed that rather than BMM media the liquid digestate could be used and this decreased water demand, additional nutrient requirement cost significantly.

Yang et al. (2011) compared the water demand and nutrient demand when wastewater, seawater and freshwater used to cultivate *Chlorella vulgaris* in raceway to obtain biodiesel. They declared that to generate 1 kg biodiesel, 3726 kg water, 0.33 kg nitrogen, and 0.71 kg phosphate were required if freshwater used without recycling. Recycling harvest water reduced the water and nutrients usage by 84% and 55%. When they used sea and wastewater, they decrease 90% water requirement and eliminated the need of all the nutrients except phosphate. Using sea/wastewater for algal culture could reduce nitrogen usage by 94% and eliminated the need of K, Mg and S. Overall, using seawater or wastewater as the culture medium could reduce the water footprint of microalgae biodiesel production by approximately 35%.

Currently, there have been wide applications of wastewater to microalgae cultivation including municipal wastewater, domestic wastewater, urban wastewater (Guldhe et al, 2017), anaerobic digestate (Guldhe et al, 2017; Zhang et al, 2017), leachate (Garcia et al, 2017) and urine (Crofcheck et al, 2012; Tuantet et al, 2014). Except food industries, many Industrial wastewaters are not suitable for large scale microalgae cultivation due to their high SS and heavy metals content and dark color (Guldhe et al, 2017). Digestate is a kind of wastewater and contains nutrients, solids and

microorganisms. It is also used for microalgae cultivation and this process will be given in detail “Digestate” part.

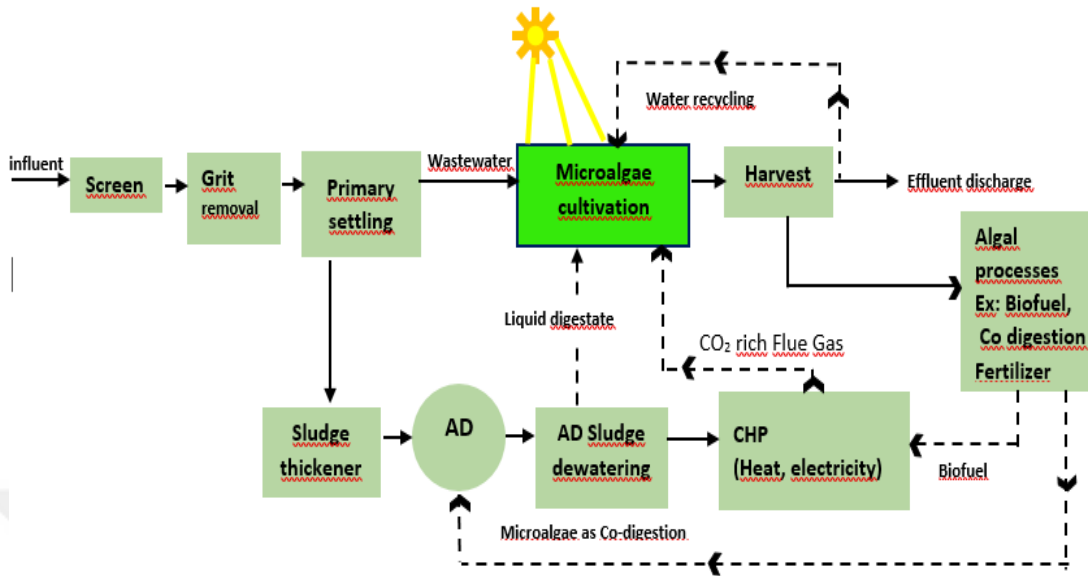


Figure 2.5 : Microalgae cultivation in wastewater. Modified from Bohutskyi et al (2017).

2.3.1 Anaerobic digestion

Anaerobic metabolism is the metabolism in which oxygen plays no part. By the help of this metabolism organic matter is converted into biogas that is used as energy source. Basically, in anaerobic decomposition of organic matter, complex organic compounds are fermented to low molecular weight fatty acids (volatile acids). Then, the low molecular organic acids are converted to methane. Anaerobic degradation of organic matter is also expressed as “Anaerobic Digestion”. Anaerobic Digestion is generally divided into 4 main groups (Figure 2.6). These are; hydrolysis stage in which polymers are converted into monomers; fermentation (acidogenesis) stage in which monomers are converted into VFAs; acetogenesis stage in which VFAs converted into acetate and Formate+H₂; methanogenesis stage in which acetate and Formate+H₂ are converted into methane which provides energy. At the end of Anaerobic Digestion, biogas is produced as an electricity and heat source. Biogas contains of 50–65% CH₄, 30–45% CO₂, and other trace gases like H₂S, H₂ and water vapor, nitrogen gas (N₂) and ammonia (NH₃) (Selvaraj et al, 2017; Uggetti et al, 2017). These percentages in biogas can be variable based on the substrate(feedstock) used in anaerobic digestion.

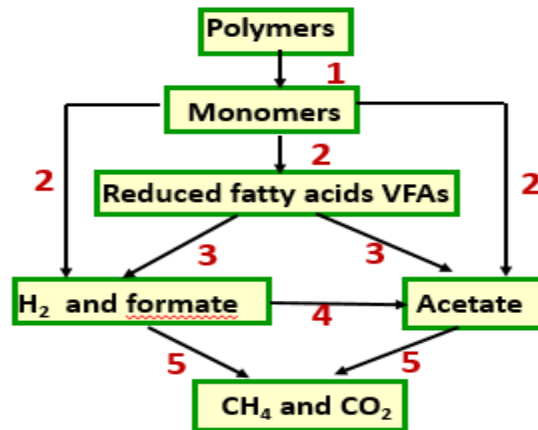


Figure 2.6 : Anaerobic digestion steps. 1 is hydrolytic microorganisms (Hydrolysis stage); 2 is fermentative microorganisms (Acidogenesis stage); 3 is acetogenetic bacteria (Acetogenesis); 4 is homoacetogenic bacteria ;5 is methanogenetic bacteria (Methanogenesis).

Currently, either the residue of microalgae species or microalgae biomass directly are used as feedstock for anaerobic digestate to produce biogas. When microalgae biomass is used lipid extraction to obtain biofuel, the residue of microalgae is still containing carbohydrates and proteins which could undergo anaerobic digestion to produce biogas (Uggetti et al, 2017). For example, Yang et al. (2011) obtained a methane yield of 0.39 L CH₄/gVS by digesting residual *Scenedesmus* biomass derived from oil extraction processes. The thing must be considered of using microalgae residue after lipid extraction is the solvents used for the transesterification process. Because some solvents can be reduced the methane production (Uggetti et al, 2017).

The residue of microalgae can be also used as co-digestion (Figure 2.5). Microalgal biomass generally contains high amounts of nitrogen, hence very low C/N ratios around. Therefore, carbon-rich co-substrates may be added to enhance the methane conversion process. For example, Yen & Brune (2007) mixed residual *Scenedesmus* sp. and *Chlorella* sp. with carbon-rich paper waste and obtained doubled methane yield from 0.14 to 0.23 L CH₄/g VS compared to carbon-rich paper waste itself.

2.3.2 Liquid digestate

At the end of anaerobic digestion, the liquid effluent is called as either “Anaerobic Digestate” or “Digestate”. It is a mixture of stabilized influent and microbial biomass (Uggetti et al, 2017). As already mentioned, during the anaerobic process, part of the organic matter is converted into methane; therefore, the organic content of the

digestate is lower than in the influent. Although the organic content of the digestate is lower than in the influent, digestate still has a high carbon content, and liquid digestate contains significant amounts of N, P, and total solids (Liu et al, 2016).

The substrates (feedstock) commonly used in anaerobic digestion include manure, agricultural waste, energy crops (residual microalgae or microalgae itself), waste from food-processing industries, and organic fraction of municipal waste (including sewage sludge). The type of substrate used not only determines the amount of biogas produced, but also the amount of digestate and its content of plant nutrients.

The digestate can be used as fertilizer directly by reducing the cost of synthetic fertilizer. However it can be used for directly as fertilizer, because of its solid content it can damage soil quality. For this reason, digestate generally separated into liquid and solid phase. Liquid phase is used for fertilizer; but damaging soil quality might be occurred when liquid phase is also used (Vaneckhaute et al, 2017). Microalgae can be cultivated liquid phase of digestate of digestate itself with variable dilutions (Table 2.2). Recent studies focus on microalgae cultivation in variable digestate to obtain valuable products like biofuel. In addition, the residual microalgae is either used for fertilizer or other processes like co-digestion to produce biogas. The liquid phase is discharged with membrane. But there are some challenges.

The growth of microalgae can be inhibited by high nutrients concentrations (NH_4) and suspended solids and COD. 200 mg $\text{NH}_4\text{-N/L}$ or higher concentrations can inhibit microalgae growth. COD concentration higher than 1100 mg/L. may also inhibited microalgae cultivation (Abu-Hajar et al, 2017). Suspended solids hinders the light penetrate in the digestate liquid that inhibits microalgae cultivation in terms of photosynthesis process. At this case, dilute the digestate and its dilution ratio is very important issue to cultivate microalgae.

Wang et al. (2010) examined different dilution multiples of 10, 15, 20, and 25 were applied to the digested manure and algal growth was compared in terms of the growth rate. Slower growth rates were observed with less diluted manure samples with higher turbidities in the initial cultivation days. At the end of the experiments 100% $\text{NH}_3\text{-N}$, 70.1 %TP, 82%. TKN, 38% COD removals in 10% dilution; 100% $\text{NH}_3\text{-N}$, 62% TP, 75% TKN, 27%COD removals in 15% dilution; 100% $\text{NH}_3\text{-N}$, 71 % TP, 78 % TKN, 34 % COD removals in 20% dilution, 100% $\text{NH}_3\text{-N}$, 74 % TP, 77 % TKN, 27 % COD

removals in 20% dilution were determined. 1.57; 1.47; 1.71; 1.48 g biomass L⁻¹ were obtain for 10;15; 20; 25 dilutions respectively. The highest biomass was shown in 20 dilutions although its removal efficiency values are lower than %10 percent. This is explained as nutrient effect. Although light penetration is lower than 10 percent because of the nutrients abundancy biomass concentration found highest. But it is recommended to use 10% percent by adding suitable nutrients to prevent ammonia inhibition.

Bjornsson et al. (2013) did experiments with the digestate of swine manure and diluted it with distilled water until they reach variable NH₃-N and PO₄-P concentrations. They obtained high biomass and nutrient removal efficiency when they added additional nutrients without supplemental CO₂ addition. At first they did not add nutrient into swine manure and determined 57 %NH₃-N and 45% PO₄-P removal efficiency and 0.14±0.04 g biomass L⁻¹. When they added MgSO₄ and MgCl into solution, 99.9% NH₃-N, 65.2% PO₄-P and 92% NH₃-N, 82.8% PO₄-P removals were determined respectively. 0.33 and 0.4 g biomass L⁻¹ were found respectively. Then, they added %25 algal biomass and %50 algal biomass into solution and 99% NH₃-N; 89 %PO₄-P and 99% NH₃-N; 81 %PO₄-P removals were determined respectively. 0.37 g biomass L⁻¹ obtained in digestate solution containing %25 algal biomass ; 0.42 g biomass L⁻¹ obtained in digestate solution containing 50% algal biomass. Due to the fact that algal biomass contains nutrients like Mg, the biomass productivity was high. Mg itself has high effect on biomass.

Although dilution is obligatory to cultivate phototrophic microalgae in the digestate, diluting the digestate too much can also affected negatively biomass concentration of microalgae in terms of nutrient deficiency. For instance, Abu Hajar et al. (2017) did experiments with the digestate containing food waste and animal manure and diluted it with 0.05, 0.10, 0.30, 0.60, 1.25, 2.5, 3.75, 5, 7.5 and 10 % dilutions ratio respectively. The highest biomass concentration found in 10% dilution. At this point nutrient deficiency can be considered as negative effect on microalgae growth. Because there is less nutrients in 0.05, 0.10, 0.30, 0.60, 1.25, 2.5, 3.75, 5, 7.5 % dilutions compared to 10 % dilution with 0.667 mg/l biomass concentration as it is seen in Table 2.1.

Table 2.1 : Microalgae sp. cultivation and their biomass concentration in different digestate.

Digestate	Microalgae species	Metabolism of Microalgae	Reactor type	Biomass (g dw L ⁻¹)	References
Food waste and animal manure	<i>Scenedesmus dimorphus</i>	Photoautotrophic Mixotrophic	Batch, Lab scale	0.667	Abu Hajar et al, 2017
Swine manure	<i>Scenedesmus sp.</i> AMDD	Photoautotrophic	Batch, Lab scale	0.42	Bjornsson et al, 2013
Cattle manure	<i>Scenedesmus sp</i>	Photoautotrophic	Batch, Lab scale	-	Scherer et al, 2017
Sewage sludge	Mixed microalgae <i>Scenedesmus sp.</i> dominant	Photoautotrophic Mixotrophic	Batch, Lab scale	2.6	Sialve et al, 2014
Diary manure	<i>Chlorella sp</i>	Photoautotrophic, Mixotrophic	Batch, Large scale	1.57	Wang et al, 2010
Sewage sludge				0.268	

Table 2.2 : Characterization of various digestates in which microalgae cultivated.

Digestate type	pH	mg/L							References
		TS	TN	NH ₄ -N,	TKN	TP	PO ₄ -P	COD	
Cattle Manure	7.5	36900±300	2313	893	2.24%	1119	-	42600±3500	Go'mez et al, 2011; Kobayashi et al, 2013
DWW sludge- (ADC)	6.9– 7.2	2950±320	880±65	800±100	-	51±10	40±7	1600±400	Bohutskyi et al, 2016
Diary manure		-	1782		3305	266	-	38230	Wang et al, 2010
Cow manure	7.76	15000	-	1500	2960	1330	-	15500 6800*	Li et al, 2014.
Swine Manure	8.5	17620	3355	2800	3500	-	318	17600	Lencioni et al,2016; Franchino et al, 2016
OFMSW	8.4	-	-	2800	4500	-	130	7300	Quina et al, 2015; Tampio et al, 2016

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Culture media

In this work BG11 medium used as culture media to grow mixed Microalgae sp.

Composition of BG11 medium given in Table 3.1. and Table 3.2.

Table 3.1 : The composition of BG-11 medium.

Ingredient	Concentration	
	g. L ⁻¹	mM
NaNO ₃	1.5	17.67
K ₂ HPO ₄ . 3H ₂ O	0.04	0.18
MgSO ₄ . 7H ₂ O	0.075	0.30
CaCl ₂ . 2H ₂ O	0.036	0.25
Citric acid	0.006	0.029
Ferric ammonium citrate	0.006	0.030
EDTA	0.001	0.0024
Na ₂ CO ₃	0.04	0.38
Trace Metal Solution	Add 1mL	
Deionized water	To 1 L	

Table 3.2 : The components of Trace Metal Solution.

Ingredient	Concentration
	g. L ⁻¹
H ₃ BO ₃	2.86
MnCl ₂ . 4H ₂ O	1.81
ZnSO ₄ . 7H ₂ O	0.222
Na ₂ MoO ₄ . 2H ₂ O	0.390
CuSO ₄ . 5H ₂ O	0.079
Co(NO ₃) ₂ . 6H ₂ O	0.0494
Deionized water	To 1 L

3.1.2 Chemicals

Chemical used in this work given in Table 3.3. In this study, buffer HEPES solution was added to each sample for maintaining pH between 7 and 7.5. HEPES solution is added as 10 mL for 1L solution.

Table 3.3 : The chemical used in this experiment.

Chemical	Company
HEPES	Sigma Aldrich
Ethanol	Merck
Methanol	Merck
H ₃ BO ₃	Merck
MnCl ₂ . 4H ₂ O	Merck
ZnSO ₄ . 7H ₂ O	Merck
Na ₂ MoO ₄ . 2H ₂ O	Merck
CuSO ₄ . 5H ₂ O	Merck
Co(NO ₃) ₂ . 6H ₂ O	Merck
NaNO ₃	Merck
K ₂ HPO ₄ . 3H ₂ O	Merck
CaCl ₂ . 2H ₂ O	Merck
Citric acid	Merck
Ferric ammonium citrate	Merck
EDTA K ₂ Mg. 2H ₂ O	Merck
Na ₂ CO ₃	Merck

3.1.3 Laboratory equipment

Laboratory Equipment used in this work given in Table 3.4:

Table 3.4 : List of equipment used in this work.

Laboratory Equipment	Firm / Model
Micropipettes	Biohit, m10, m100, m1000, m5000
pH-meter	Hanna
Eppendorf Thermomixer	Thermomixer Compact
Fridge (4°C)	-
Vortex	Labnet
UV-Vis Spectrophotometer	Bio-Rad SmartSpec Plus
Oven 103-105°C	-
Centrifuge (big)	Hettich Rotina
Centrifuge (small)	Hettich Rotina 38R
Oven 550 °C	Carbolite
Microscope	Leica TCS SP5 X
Analytical weight	-
Water Bath	-

3.2 Methods

In this study, the aim is to increase biomass of the mixed microalgae cultured in digestate by adding various nutrients based on BG11 media. The mixed microalgae culture was taken from Tubitak Project No: 115Y589. The Digestate is from Biosun Pamukova Katı Atık İşleme Enerji ve Çevre Sanayi Ticaret A.Ş. located at Pamukova (Sakarya). The mixed culture cultivated in BG11 media under in the cabinet as it seen in Figure 3.1. with 24 h of continuous white led light with intensity of $120\text{--}200\ \mu\text{E}/\text{m}^2\cdot\text{s}^{-1}$ at $25\ ^\circ\text{C} \pm 2^\circ\text{C}$. When inoculum is ready, 600 ml batch cultures were prepared contained digestate with 1:10 dilution ratio. Different nutrients were added to the each culture and microalgae growth was observed by TSS, chlorophyll measurement and cell counting techniques. Additionally, their $\text{NH}_3\text{-N}$, organic C and organic nitrogen, $\text{PO}_4^{3-}\text{-P}$ removal efficiencies were also determined by using standart methods whether there was an affect of removal efficiency or not. Control group was anaerobic digestate effluent with mixed culture without adding nutrient. Experiments were continued until they reached stationary phase. Water loss due to evaporation was made up daily by adding tap water before sampling. Summary of the methods' step is shown in Figure 3.2.



Figure 3.1 : Culturing Cabinet.

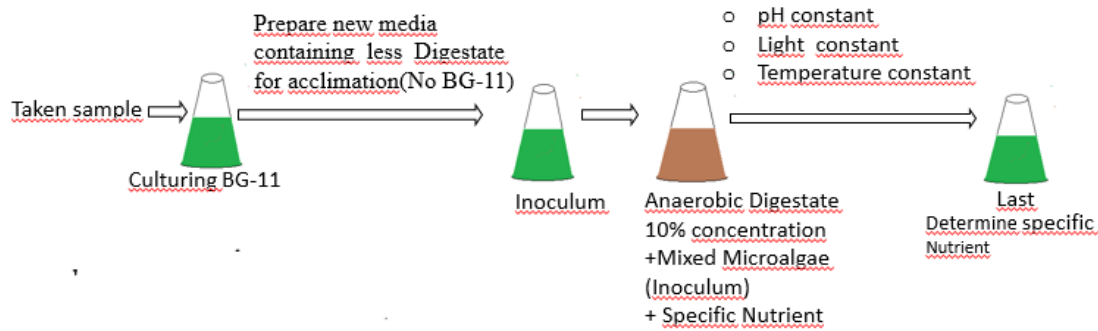


Figure 3.2 : The steps of methods in a nutshell.

3.2.1 Chlorophyll measurement

Chlorophyll measured based on method by Aslan & Kapdan, 2006. 2 ml samples taken and put into eppendorf tubes. They are centrifuged with 9000 rpm for 10 minutes. Supernatant of the samples discarded and added 600 ul methanol each. Vortex was used to obtain homogenous solution. After vortex they put into eppendorf thermomixer machine to heat them 80°C for 5 minutes. After heating, 400 ul methanol added to samples each and they are centrifuged 10000 rpm for 5 minutes. Supernatant of the samples are taken and put into cuvettes. They measured in spectrophotometer with the wavelength of 665 and 650nm. As seen in equation 3.1, the total chlorophyll content was determined:

$$\text{Chlorophyll a (mg/L)} = (16.5 \times 665_{\text{nm value}}) - (8.3 \times 650_{\text{nm value}}) \quad (3.1)$$

3.2.2 Samples having additional nutrient

1 L flasks containing %10 digestate concentration prepared first, then inoculum was added each. Based on BG11 media, Mg, P, Fe, Ca sources and EDTA elements were added to prepared samples each. Some samples had double concentration and more to decide which one was the best.

Samples put into cabin under 24 h of continuous white led light with intensity of 120-200 $\mu\text{E}/\text{m}^2\cdot\text{s}^{-1}$ at $25^\circ\text{C} \pm 2^\circ\text{C}$ temperature, pH and light were constant. Each bottle's growth rate, and removal efficiency of N and P was determined.

3.2.3 Cell counting

For cell counting “Thoma lam” was used. It is a special lam and generally used for yeast and sperm counting and it is not suitable for bacteria counting. It has 16 squares and the total volume of 16 squares 0.1 mm^3 as it is seen in Figure 3.3. During the experiments, all cells were counted and cell numbers were determined as the equation 3.2 given below:

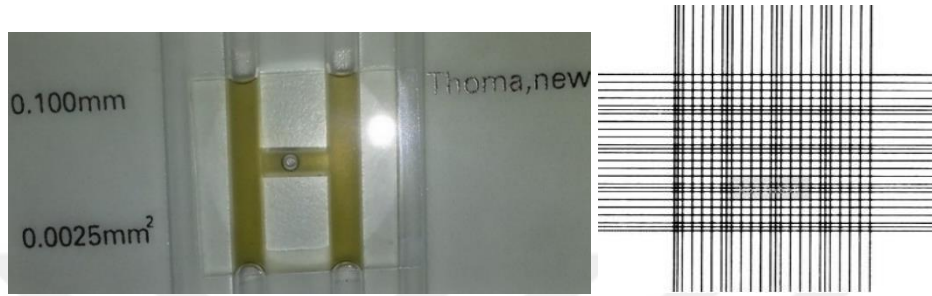


Figure 3.3 : Thoma lam used in this study and its 16 squares (kocintok.com).

$$\text{Cell/cm}^3 = A \times DF \times 10000 \quad (3.2)$$

Where A was cell number in 16 squares; DF was dilution factor; 10000 was unit conversion for 1 cm^3 .

3.2.4 Growth rate and doubling time determination

A typical growth curve shows a lag phase, an exponential or log phase, a stationary or plateau phase and decay or death phase. In the stationary phase, growth is likely limited by resources such as light or nutrients. In this study, experiments got stopped when microalgae reached the stationary phase. Growth rate ($\mu \text{ d}^{-1}$) was calculated with the following equation 3.3 in terms of the cell counting technique (Chiu et al, 2008):

$$\mu = \frac{\ln X_i - \ln X_0}{t_i - t_0} \quad (3.3)$$

Where X_0 was initial cell number value for t_0 ; X_i was final cell number value for t_i .

The doubling time for microalgae, exponential growth area's growth rate value was used as it was seen in equation 3.4 (Delgadillo et al, 2016):

$$td = \frac{\ln 2}{\mu} \quad (3.4)$$

Where $\ln 2$ was 0,6931.

3.2.5 Standard methods

TSS, VSS, TKN, NH₃-N, COD and PO₄³⁻-P measurement were done based on Standard Methods. These were for the characterization of the digestate and determining whether there was removal of either nutrient or organic matter or not. TKN showed organic N amount + NH₃-N in the samples, NH₃-N showed soluble N amount, COD showed total organic content, PO₄-P showed soluble inorganic phosphorus. All measurements and sample frequency were shown in Table 3.5.

Table 3.5 : Sample Frequency.

Parameter	Sample Frequency
Chlorophyll Measurement	Once in 2 days
Optical Density	Once in 2 days
Cell Counting	Once in 2 days
Total Suspended Solids	Initial and Final
Ammonia Nitrogen NH ₃ -N	Initial and Final
TKN (Total Kjeldahl Nitrogen)	Initial and Final
Ortophosphate PO ₄ ³⁻ -P	Initial and Final
Chemical Oxygen Demand	Initial and Final

Removal efficiency was calculated as the formula given in equation 3.5.

$$Re = \frac{(S_o - S) * 100}{S_o} \quad (3.5)$$

Where S_o was the initial concentration; S was the final concentration.

Specific nutrient removal rate was calculated with the formula given in equation 3.6:

$$Ri = \frac{S_o - Sf}{ti - t0} \quad (3.6)$$

Where S_o was the initial concentration for $t0$; Sf was the final concentration for ti ; unit was mg/l/d.

3.2.5.1 TKN and NH₃-N measurement

Chemicals prepared for both TKN and NH₃-N measurements were borate buffer solution, dechlorinating reagent (sodium thiosulfate- Na₂S₂O₃·5H₂O), absorbent solution, plain boric acid, standard sulfuric acid titrant (0.02 N), digestion solution (K₂SO₄, CuSO₄ and H₂SO₄ containing).

TKN measurement was done based on Standard Method 4500-N_{org} (APHA, 2005). Taken sample was digested with 50 mL digestion solution under the heater. When white fume was seen, it was waited until sample become transparent and pale green. Pale green color showed digestion's end. After digestion, samples got cool and dilute with 300 mL distilled water then mixed. Then, sodium hydroxide- thiosulfate reagent (Neutralization agent) added to sample carefully. Then add 25 mL borate buffer solution and adjust pH 9.5 with 6N NaOH using. Lastly sample and 50 ml Boric acid solution were inserted distillation apparatus like it was done for NH₃-N measurement and called "distillation".

Distillation for NH₃-N measurement (Standard Method 4500-N_{NH3} of AWWA, 2012 for NH₃-N), add 25 mL borate buffer solution and adjust to pH 9.5 with 6N NaOH. Then, assemble the sample flask and 250 mL erlenmeyer flask containing 50 mL indicator boric acid solution to the distillation apparatus. Continue the distillation until 250 ml distillate collected and boric acid's plain lavender color changed into green. Then start titration. Titrate the distillate with standard 0.02 N H₂SO₄ titrant until green color changes into pale lavender. Blank was distilled water. Expression of results was seen in equation 3.7 and 3.8.

$$\text{Total Khejldahl Nitrogen (mg/L NH}_3\text{-N)} = \frac{(A-B) \times N \times 14 \times 1000}{V_{\text{sample}}} \quad (3.7)$$

$$\text{mg/L NH}_3\text{-N} = \frac{(A-B) \times N \times 14 \times 1000}{V_{\text{sample}}} \quad (3.8)$$

Where A was volume of H₂SO₄ titrated for sample, mL; B was volume of H₂SO₄ titrated for blank, mL; N was normality of H₂SO₄ and 1000 was unit conversion

3.2.5.2 COD measurement

The chemical oxygen demand (COD) was quantifying the organic matter content of a sample by acidic digestion with potassium dichromate solution at acidic pH and in the presence of silver catalyst. In this study the COD test was run at 150°C for 2 hours in an open reflux arrangement. Chemicals prepared for COD measurement were standard K₂Cr₂O₇ (digestion) solution (0.25 N), ferroin indicator solution, acid catalyst solution (Ag₂SO₄ and H₂SO₄ containing), ferrous ammonium sulfate solution (FAS; 0.12 N).

Experimental Procedure: 10 mL sample was taken into an erlenmeyer flask and 5 mL dichromate reagent added into sample. Then, carefully and slowly add 15 mL acid

solution to the flask. Immediately the samples pulled under hot plate and connect the flask to the condenser to start digestion of samples for 2 hours. At the end of 2 hours, the heater was turned off. After 45 min, the insides of the condensers were thoroughly rinsed with 45 mL distilled water to gather volatiles organics stuck to the condenser glass. Then, the flasks were cooled down to room temperature. For sample titration, 2-3 drops of ferroin indicator were added to the sample flask and titrated with FAS solution until the color turned from greenish blue to dark red. For blank, 10 mL distilled water was treated in the same manner as indicated above in the experimental procedure to eliminate possible interferences. Results were expressed as mg/L COD and calculation of results are seen in equation 3.9.

$$\text{mg COD/L} = (A-B) \times C \times 8 \times 1000 / D \quad (3.9)$$

Where A was titrant consumption for the blank (mL), B was titrant consumption for the sample (mL), C was normality of FAS titrant (N), D was sample volume of (mL).

3.2.5.3 Orthophosphate ($\text{PO}_4^{3-}\text{-P}$) measurement

The purpose of this experiment is to determine the dissolved orthophosphate concentration in an aqueous sample colorimetrically by the stannous chloride method in accordance with Standard Methods 4500-P (APHA, 2005).

Add 1 drop phenolphthalein indicator to 100 mL sample containing. If the sample turned pink, add 4.0 mL molybdate reagent as the strong acid solution to remove the color. Then add 0.5 mL (10 drops) stannous chloride solution. Make sure that the temperature of the reagents and samples were constant and between 20°C and 30°C throughout the experiment because temperature affected both the rate and intensity of color development. After 10 min but before 12 minutes record the absorbance (color) at 690 nm wavelength in 1 cm path-length cuvette and compare with a calibration curve, using distilled water as the sample blank. The calculation formula was given in equation 3.10.

$$\text{Phosphate concentration (mg/L } \text{PO}_4^{3-}\text{P)} = \text{ug P /ml } V_{\text{sample}} \quad (3.10)$$

$$\text{ugP} = \text{mgP}/1000$$

3.2.5.4 TSS and VSS measurement

TSS concentration in samples were measured based on Standard method 2540-D (APHA , 2005). Glass fiber filters washed 20 mL distilled water three times and dried 550 °C over for 30 minutes for preparation. Then the ready filter was inserted filtration apparatus. The sample transferred through into the filter volumetric pipette. The filter dried at least one hour at 103-105° C. Cool in a desiccator until constant weight was reached. The formula was given in equation 3.11

$$\text{Total Suspended Solids (TSS), mg/L} = (A-B) \times 1000 / V_{\text{sample}} \quad (3.11)$$

Where, A was weight of filter and residue (mg); B was the weight of filter (mg).

For the VSS measurement, the filters containing residue put into oven 550°C for 30 minutes and then, it was cooled in a desiccator until constant weight was reached. The formula was given in equation 3.12.

$$\text{Volatile Suspended Solids (VSS), mg/L} = (A-B) \times 1000 / V_{\text{sample}} \quad (3.12)$$

Where, A was weight of filter and residue (mg); B was the weight of filter (mg).

3.2.6 Mass balance- stoichiometry for photoautotrophic systems

The equilibrium between the forms of NH_4^+ and free NH_3 depends mainly on the pH and temperature. pH values higher than 9.25 and at 25 °C, it's assumed that all NH_4^+ is converted into NH_3 form in any system. But NH_3 can leave in any system at any temperature and pH value. The calculation of the amount NH_3 leaving any system is calculated with the formula as it is given in equation 3.13.

$$[\text{NH}_3] = \frac{TAN}{\left(1 + \frac{[H^+]}{K_a}\right)} \quad (3.13)$$

Where $K_a = 5.75 \times 10^{-10}$ at 25°C.

In this study microalgae culture was photoautotrophic and the general formula of the microalgae was $\text{C}_{106}\text{H}_{263}\text{O}_{110}\text{N}_{16}\text{P}$. The stoichiometric relationship of the microalgae based on NH_4 as N source was described in the equation 3.14 and its mass balance is given in Table 3.6(Ebeling et al, 2006) :



Table 3.6 : Mass Balance of Microalgae.

Input						Output	
	NH ₄ (g)	CO ₂ (g)	H ₂ O (g)	HCO ₃ (g)	HPO ₄ (g)	Microalgae (g)	O ₂ (g)
1 mol	18	44	18	61	96	=	-
Total	288	4048	1656	854	96		3550 3392
			Total	6942		Total	6942

Based on equation 3.13 when 1 g NH₄-N was used to produce algal biomass, 18.07 g CO₂ was consumed and 15.14 g O₂ was produced. 1 g NH₄-N utilization was resulted as 15.85 g VSS algae as it was seen in equation 3.15 (Ebeling et al, 2006):

$$VSS_{\text{Algae produced}} = 15.85 \text{ g VSS algae} / \text{g N} \quad (3.15)$$

In this study the loss of ammonia nitrogen was calculated with equation 3.12. Theoretical VSS value estimated with equation 3.14. Based on the comparison of the theoretical VSS and experimental VSS values, it was tried to understand whether there was TKN conversion to NH₃-N or not.

3.2.7 Elemental composition of the digestate

The mineral concentration was measured with Atomic Absorption Spectrophotometer (AAS). Digestate was first put into centrifuge to eliminate particulates. After centrifuge, it was filtered with 0.22 µm filter as preparation method. For AAS, firstly standard solutions of the each element was measured to make a calibration curve for each. Then, the digestate was diluted with 1/50 ratio to eliminate its color effect. The elemental composition of the digestate was determined based on the calibration of the standard solutions of the elements used.

4. RESULTS AND DISCUSSION

4.1 Wastewater Characterization

The mineral concentration of the digestate was shown in Table 4.1.

Table 4.1 : The mineral composition of the digestate.

Mineral	(mg/l)	Standard. Deviation
Ca	58.13	0.22
Mg	19.06	0.035
Fe	27.79	0.013
Mn	3.86	0.082
Al	9.474	0.487
Si	46.72	0.435
Pb	1.152	0.050
B	5.839	0.021
Cr	5.279	0.0066
Cu	4.051	0.0392
Cd	0.387	0.0068
Ni	3.075	0.0063
Ag	4.834	0.0019
S	619.7	4.74
Zn	0.885	0.0183
Sr	1.039	0.0006
Na	871.4	3.59

Characterization in Table 4.1. was done by AAS. AAS did not give information about the formation of elements. For instance, Na was in high concentration and it was not known that it was formed as Na_2SO_3 or NaCl (salinity) in the digestate. Paliwal et al. (2017) declared that high NaCl concentration like 400 mM NaCl suppressed growth and had an effect on increasing lipid concentration. Again, Srivastava et al. (2017) carried on experiments to investigate salinity effect and they added 20 g/L NaCl and 25g/L NaHCO_3 into a media in which microalgae *Desmodesmus abundans*

cultivated. Based on Paliwal et al. (2017) study and Srivastava et al. (2017) study the concentration of Na in the digestate seemed to be very low.

S could be as SO_4^{2-} and could be formed as MgSO_4 , $(\text{NH}_4)_2\text{SO}_4$, K_2SO_4 and $(\text{NH}_4)_2\text{MgSO}_4$ in the digestate. Taking SO_4^{2-} as S source is not harmful for microalgae. Si is toxic for many microalgae species but it's essential for some algae species like diatoms because they have silica cell membrane structure. Napan et al. (2015) carried on toxicity experiments with microalgae *Nannochloropsis salina* and they found that 0.0135 mg/L Cd, 0.04 mg/L Cr, 0.038 mg/L Cu, 0.058 mg/L Mn, 0.112 mg/L Ni, 0.039 mg/L Pb and 0.099 mg/L Zn caused toxicity when each element added to separately into cultivation media. The concentration of these elements were high in the digestate referred to Napan et al. (2015).

The characterization of the digestate is shown in the Table 4.2. Filters with 0.45 μm pore size used for the filtered digestate values.

Table 4.2 : The composition of the liquid digestate.

Raw			Filtered		
Parameter	Unit	Value	Parameter	Unit	Value
TKN	mg/L $\text{NH}_3\text{-N}$	1070	TKN	mg/L $\text{NH}_3\text{-N}$	890
$\text{NH}_3\text{-N}$	mg/L $\text{NH}_3\text{-N}$	807	$\text{NH}_3\text{-N}$	mg/L $\text{NH}_3\text{-N}$	659
pH	-	8.91	Ortophosphate	mg/L $\text{PO}_4^{3-}\text{-P}$	65
Conductivity	mS/cm	12.1	pH	-	8.90
Alkalinity	mg/L as CaCO_3	7474	Conductivity	mS/cm	11.2
Cod	mg/L O_2	14480	Alkalinity	mg/L as CaCO_3	7024
TSS	mg/L	3930	Cod	mg/L O_2	6424
Color	PtCo	45407			

4.2 Comparision Microalgae Growth in BG11 media and Anaerobic Digestate

Determing biomass concentration, linear correlation between OD and dry weight was done with the digestate with no nutrient. But OD value gave negative value most of times for the digestate eventhough there was growth as it was seen in Figure 4.1. The impresicion of the OD values were probably because of the particulate matter and dark color of the digestate. Therefore, growth rate and doubling time were determined with the help of the cell counting data. Correlation between chlorophyll concentration and cell numbers related to growth was also check and there was correlation between chlorophyll concentration and cell numbers related to growth (Figure 4.2).



Figure 4.1 : Final and initial view on the filters. Greenish color shows final, brown color shows initial

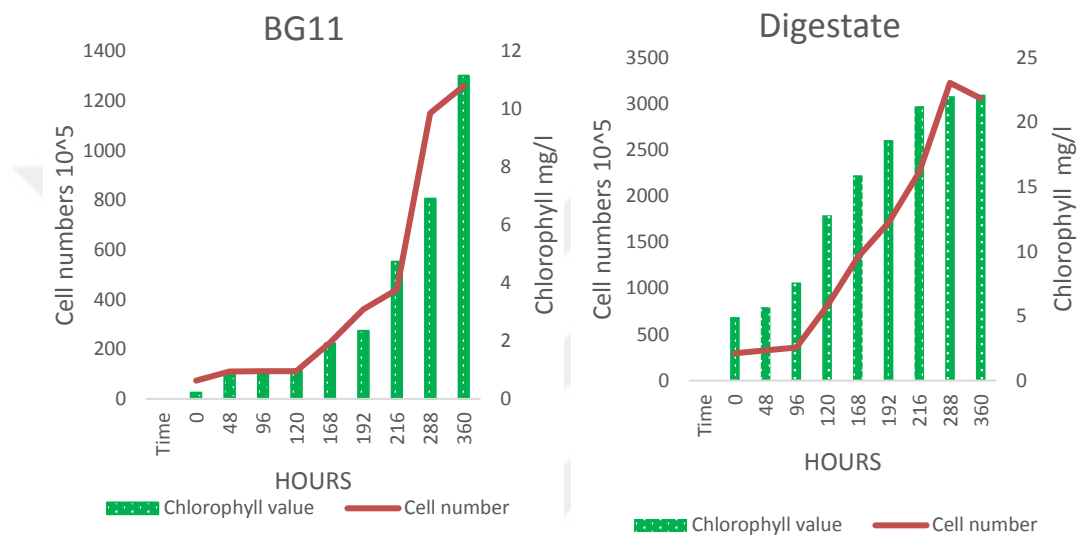


Figure 4.2 : Correlation between chlorophyll concentration and cell numbers related to growth for microalgae cultivated in BG11 and digestate separately.

The growth rate and doubling time of the BG-11 media and digestate were determined with the formulas in equation 3.3 and 3.4 by using cell counting data. The growth rate and doubling time comparison of the BG-11 media and digestate with 1:10 dilution showed in the Table 4.3. As it was expected, the growth rate and generation time were high in the BG11 media compared to digestate. This was mainly attributed to several factors. Higher turbidities of wastewater caused stress conditions, limiting light penetration and thus directly affecting to photosynthetic efficiency as it was mentioned in detail literature section. Biomass calculation were done based on VSS calculations in equation 3.12. Net biomass productivity meant subtracting initial VSS value from final VSS value. Although net biomass production of the digestate was higher than BG11, biomass increased as 3 times in the digestate. But the biomass increased approximately 8 times referred to initial value. Therefore, determining growth rate and generation time were important than determining VSS value in terms of growth.

Table 4.3 : Comparision cultures' growth rate and generation times.

Culture Media	Biomass _{ini}	Biomass _{final}	Net Biomass	μ (d ⁻¹)	TD (d)
BG11	88 mg/l	680 mg/l	608 mg/l	0.8278	0.83
Digestate (1:10)	395 mg/l	1075 mg/	680 mg/	0.1333	5.19

4.3 Magnesium

Magnesium source was MgSO₄.7H₂O. The magnesium source was added to digestate solutions in different concentrations like 0.3; 0.6; 1.2; 2.4 and 3.0 mM. Compared to the digestate, 0.3mM, 0.6 mM and 1.2 mM Mg concentration did not make a significant difference on growth. But when its concetration increased to 2.4 mM and 3.0 mM, they had more cell number and chlorophyll content compared the other solutions as seen in Figure 4.3 and 4.4 respectively. Only 3.0 mM Mg increased the growth rate and doubling time as seen in Table 4.4. Although its growth rate and doubling time were lower than 3.0 mM Mg, 2.4 mM Mg passed 3.0 mM Mg in terms of cell number, chlorophyll and biomass productivity.

Table 4.4 : The biomass concentration, growth rate and generation time of the Mg.

mM	VSS _{ini} (mg/L)	VSS _{final} (mg/L)	VSS _{net} (mg/L)	μ (d ⁻¹)	Td(d)
Digestate(1:10)	410	1200	790	0.1331	5.2
Mg 0.3	540	1290	750	0.0870	7.9
Mg 0.6	540	1350	802	0.0878	7.8
Mg 1.2	520	1300	780	0.1275	5.4
Mg 2.4	450	1320	870	0.1340	5.1
Mg 3.0	430	1275	845	0.1585	4.3

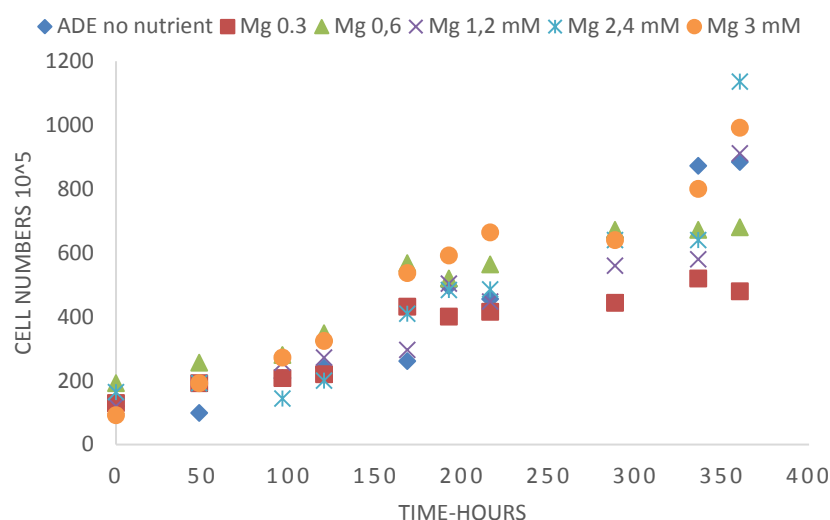


Figure 4.3 : Cell numbers of the Mg solutions.

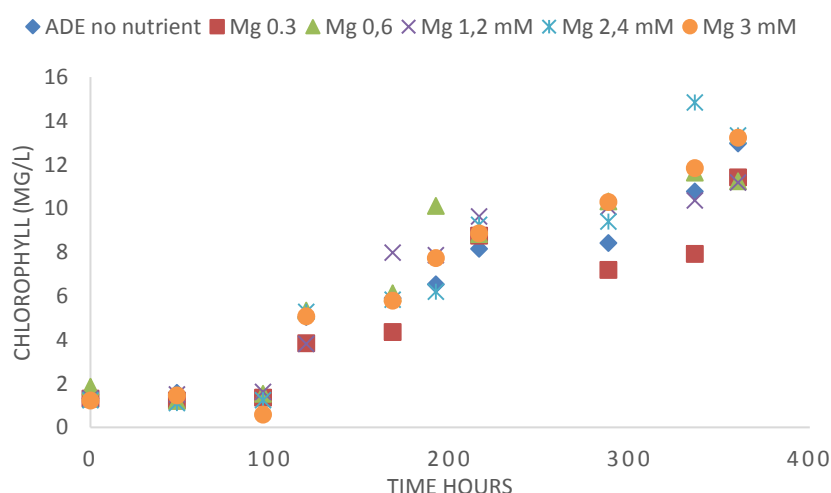


Figure 4.4 : Chlorophyll of the Mg solutions.

It was seen that Mg had positive effect on $\text{NH}_3\text{-N}$ and $\text{PO}_4\text{-P}$ removal in Figure 4.5 and 4.7 respectively. But this effect was not significant compared to the digestate and Bjornsson et al (2013) study. Bjornsson et al. (2013) did experiments with the digestate of swine manure and diluted it with distilled water. When they did not add nutrient into swine manure, the determined 57 % $\text{NH}_3\text{-N}$ and 45% $\text{PO}_4\text{-P}$ removal efficiency and $0.14 \pm 0.04 \text{ g biomass L}^{-1}$. When they added MgSO_4 , 99.9% $\text{NH}_3\text{-N}$, 65.2% $\text{PO}_4\text{-P}$ removals were determined with $0.33 \text{ g biomass L}^{-1}$. Based on the Bjornsson et al. (2013) study, it was expected to see high $\text{PO}_4\text{-P}$ removal, but it seemed that Mg had an effect on P removal negatively (in Figure 4.8).

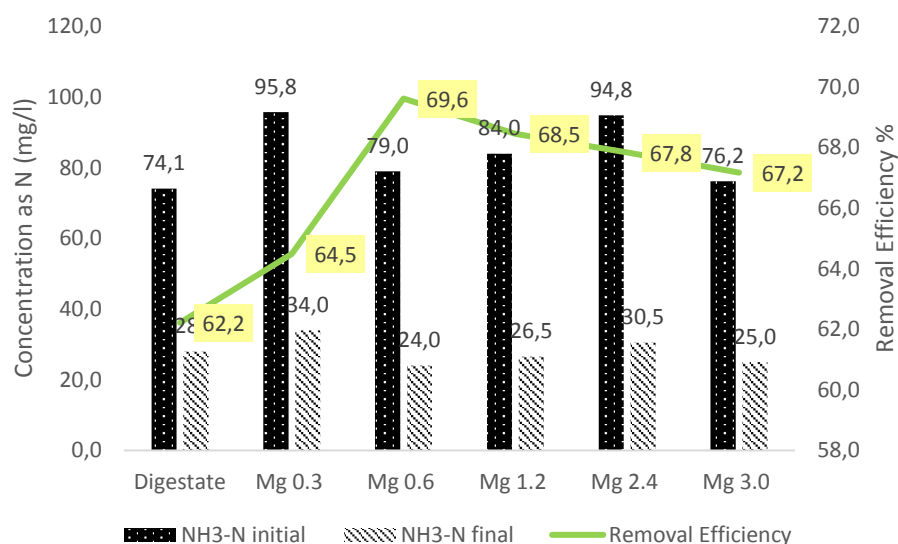


Figure 4.5 : NH₃-N removal effect of Mg.

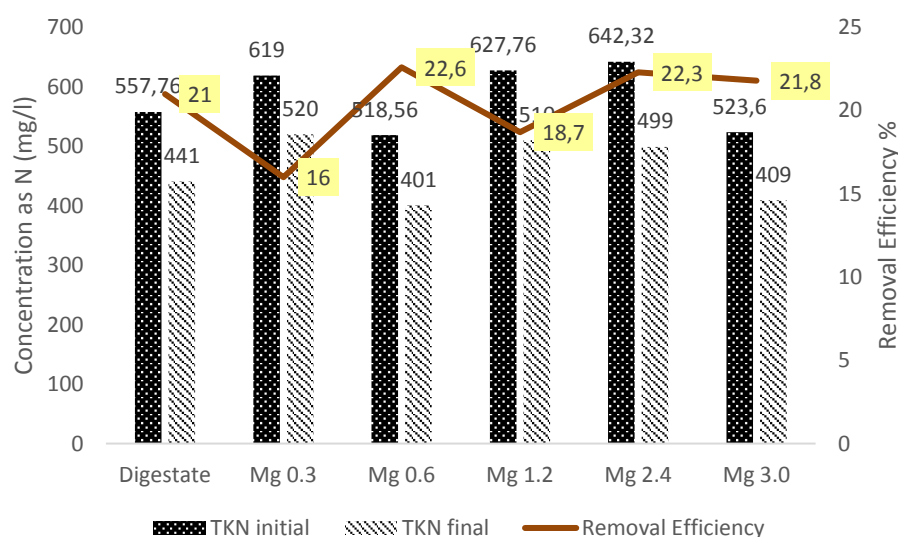


Figure 4.6 : TKN removal effect of Mg.

When pH increases NH₄ is converted into NH₃ gas form and it leaves the system (Eq 2.3). Considering this situation, TAN formula was used to determine the amount of NH₄ converted into NH₃ (Eq 3.13) based on TKN results in Figure 4.6. In this study HEPES buffer used and pH maintained between 7 and 7.5. Therefore, it was assumed that pH was 7.5 for TAN calculation. TAN calculation results for the digestate showed in the Table 4.5 TAN calculations for both NH₃-N and TKN results, the initial NH₃-N and TKN concentration were not significantly change as seen in Table 4.6 and 4.7 respectively.

Table 4.5 : TAN calculation for the digestate based on TKN experiment.

Digestate dilution	TKN _{ini} (mg/l)	Ka (25 °C)	NH ₃ -N free (mg/l)	Net TKN (mg/l)
%10	557.7	5.75*10 ⁻¹⁰	9.96	547.8

Table 4.6 : Net initial NH₄-N values for the Mg solutions based on NH₃-N results.

mM	Concentration mg/L				Theoretical VSS _{algae}	Experimental VSS _{algae} *
	NH ₃ -N ini	NH ₃ -N free	Net NH ₄ -N ini	Consumed		
Digestate	74.10	1.32	72.78	44.7	709	790
Mg 0.3	95.76	1.7	94.05	60	951	750
Mg 0.6	79.00	1.4	77.59	53.6	849	802
Mg 1.2	84.00	1.5	82.50	56	887	780
Mg 2.4	94.83	1.7	93.13	62.6	992	870
Mg 3.0	76.16	1.36	74.80	49.8	789	845

*Taken from Table 4.4.

Table 4.7 : Comparision of the theoretical biomass and net biomass production of the Mg experiment based on TKN results.

mM	Concentration mg/L				Theoretical VSS _{algae}	Experimental VSS _{algae}
	TKN _{ini}	NH ₃ -N free	Net TKN	Consumed		
Digestate	557.7	9.96	547	116	1850	790
Mg 0.3	619	11	608	99	1569	750
Mg 0.6	518.5	9.26	509	117.5	1863	802
Mg 1.2	627.7	11.1	616	117.7	1866	780
Mg 2.4	642.3	11.4	630	143.3	2271	870
Mg 3.0	523.6	9.35	514.2	114.6	1816	845

Based on Table 4.6. and Figure 4.5 consumption of the NH₄-N determined. And then, theoretical biomass production calculated with the formula shown in equation 3.15. Comparision between theoretical biomass production and net biomass production in this experiment was shown in Table 4.7. The same procedure was done for the TKN data as seen in Table 4.8. In both Table 4.7 and Table 4.8, the theoretical biomass was higher than experimental biomass for each solution Their NH₄-N removal rate based on TKN data were 7.73, 6.6, 7.83, 7.84, 9.55, 7.64 mg/l/d for the digestate, Mg 0.3 mM, Mg 0.6 mM, Mg 1.2 mM, Mg 2.4 mM, Mg 3.0 mM, respectively. The highest removal rate was Mg 2.4 mM, in contrast the highest net biomass productivity was shown in Mg 3.0 mM. COD removals in Figure 4.8 were also found low.

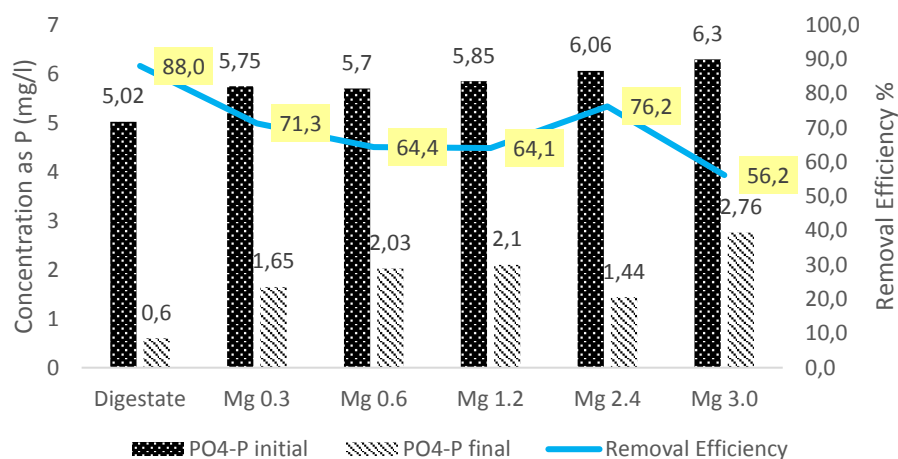


Figure 4.7 : PO₄-P removal effect of Mg.

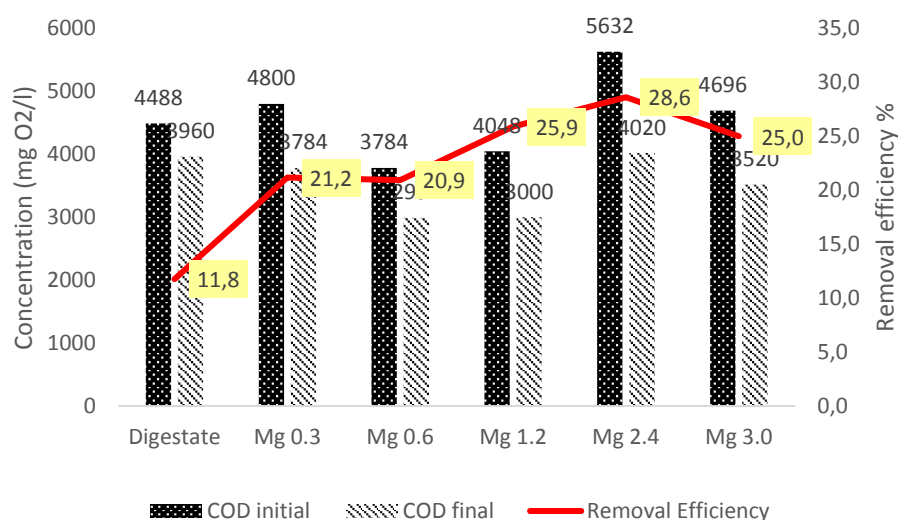


Figure 4.8 : COD removal effect of Mg.

4.4 EDTA

EDTA was added to the digestate media in different concentration each (Table 4.8). 0.0024 mM EDTA and 0.0048 mM concentration did not make a significant difference on growth when compared to anaerobic digestate. But when EDTA concentration was increased 6 times as 0.0144; and 10 times as 0.024mM, all supported growth showed in Table 4.5; in Figure 4.9 and 4.10. Although 0.0144 mM EDTA showed better values than 0.024 mM EDTA in terms of growth, 0.024mM EDTA showed better results than

0.0144 mM EDTA in terms of both growth and removals. The highest the ammonical nitrogen removal efficiency showed in EDTA 0.024 mM as 77%. Solutions net (real) $\text{NH}_4\text{-N}$ concentrations calculated with TAN formula and given in Table 4.10. Net consumed $\text{NH}_4\text{-N}$ calculated and comparison between theoretical biomass production and net biomass production in this experiment were shown in Table 4.10.

Table 4.8 : The biomass concentration, growth rate and generation time of EDTA.

mM	VSS _{initial} (mg/l)	VSS _{final} (mg/l)	VSS _{net} (mg/l)	μ (d ⁻¹)	TD (d)
Digestate	410	1200	790	0.1331	5.2
EDTA 0.0024	400	1300	900	0.1198	5.7
EDTA 0.0048	500	1470	970	0.1266	5.4
EDTA 0.0144	360	1320	960	0.1745	3.9
EDTA 0.024	410	1410	1000	0.1700	4.0

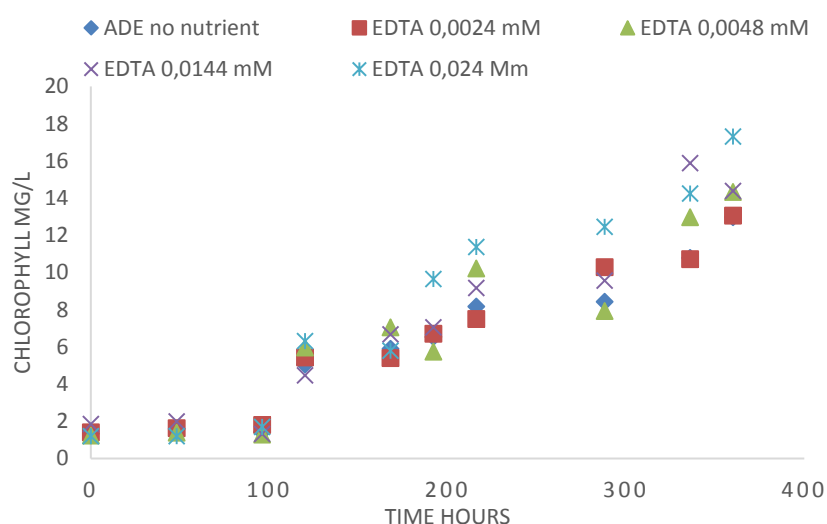


Figure 4.9 : Chlorophyll of the EDTA solutions.

In Figure 4.11 it was seen that when EDTA concentration increased, the removal of the ammonia nitrogen increased. The same situation observed in PO_4^{3-} removal in Figure 4.14. Theoretical biomass and the experimental biomass were determined based on TKN value in Table 4.10. Theoretical biomass values were higher than experimentals' as Mg results. The highest biomass was obtain in digestate media containing 0.024 mM EDTA. 7.06, 9.26, 13, 12.1 and 11.4 mg/l/d were calculated nitrogen removal rate for the digestate, 0.0024 mM, 0.0048 mM, 0.0144 mM and 0.024 mM EDTA, respectively. Based on nitrogen removal rate data, it could be said that additional EDTA had a positive effect on nitrogen removal rate. When COD removals

in Figure 4.12 and TKN removals in Figure 4.13 were checked, their removals were low and had close values like digestate solutions containing Mg source. There was no significant difference in P removals in Figure 4.14.

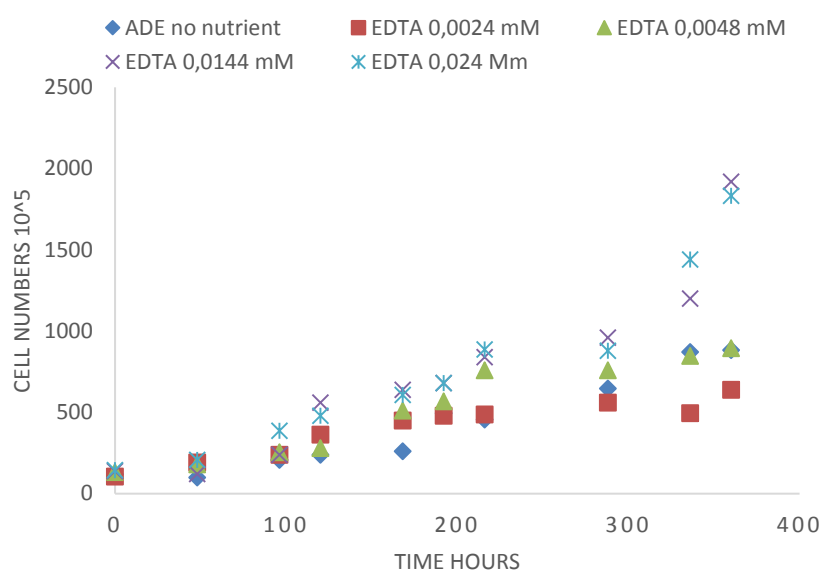


Figure 4.10 : Cell numbers of the EDTA solutions.

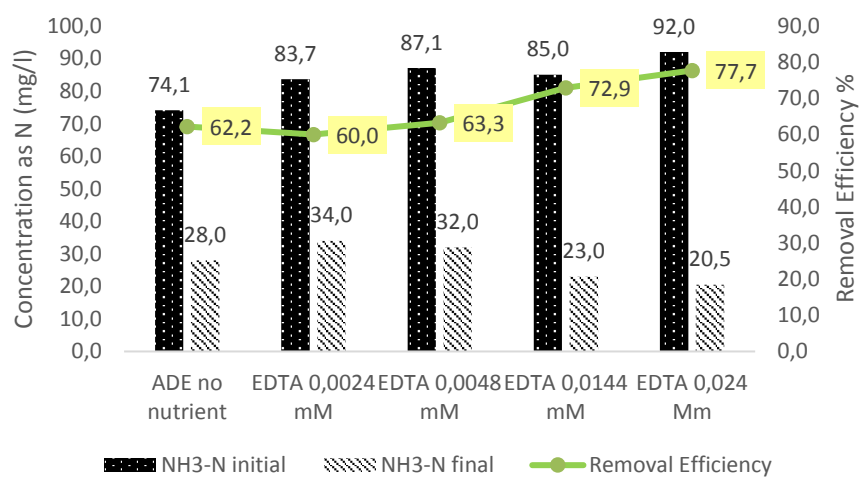


Figure 4.11 : NH₃-N removal of the EDTA solutions.

Table 4.9 : The Net initial $\text{NH}_4\text{-N}$ values for the EDTA solutions based on $\text{NH}_3\text{-N}$.

Unit (mg/L)→ Solutions mM↓	$\text{NH}_4\text{-N}$ Initial	$\text{NH}_3\text{-N}$ free	Consumed	Theoretical VSS	Experimental VSS
Digestate	74	1.32	44,78	709	790
EDTA 0.0024	83	1.5	48,21	764	900
EDTA 0.0048	87	1.5	53,52	848	970
EDTA 0.0144	85	1.5	60,48	958	960
EDTA 0.024	92	1.64	69,86	1107	1000

Table 4.10 : The Net initial $\text{NH}_4\text{-N}$ values for the EDTA solutions based on TKN.

mM	TKN_{ini}	$\text{NH}_3\text{-N}$ free	Net TKN	Concentration mg/L		
				Consumed	Theoretical VSS algae	Experimental VSS algae
Digestate	557.8	10	547	106	1692	790
EDTA 0.0024	603	10.8	592	139	2208	900
EDTA 0.0048	733	13.1	720	194	3089	970
EDTA 0.0144	733	13.1	720	182	2899	960
EDTA 0.024	575	10.3	564.8	160	2549	1000

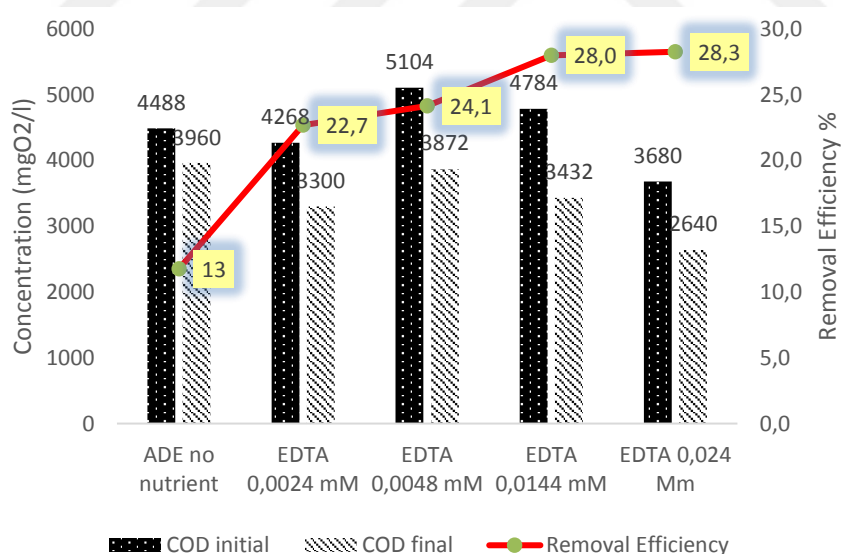


Figure 4.12 : COD removal of the EDTA solutions.

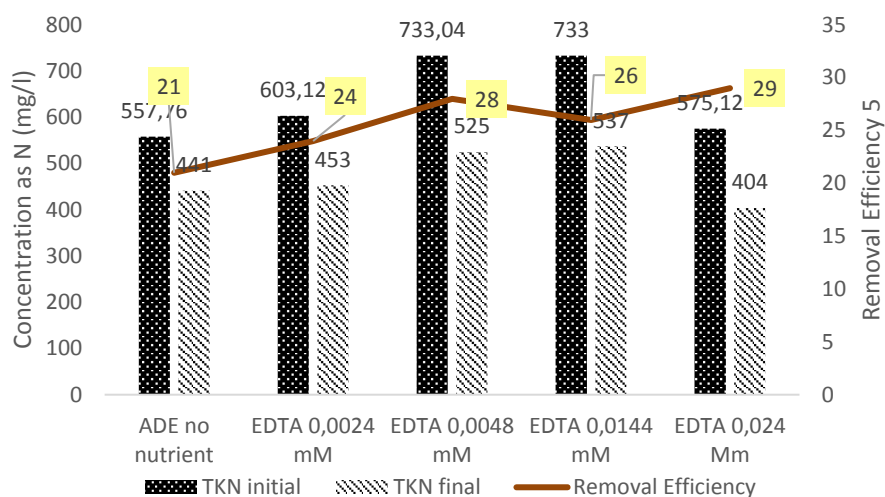


Figure 4.13 : TKN removal of the EDTA solutions.

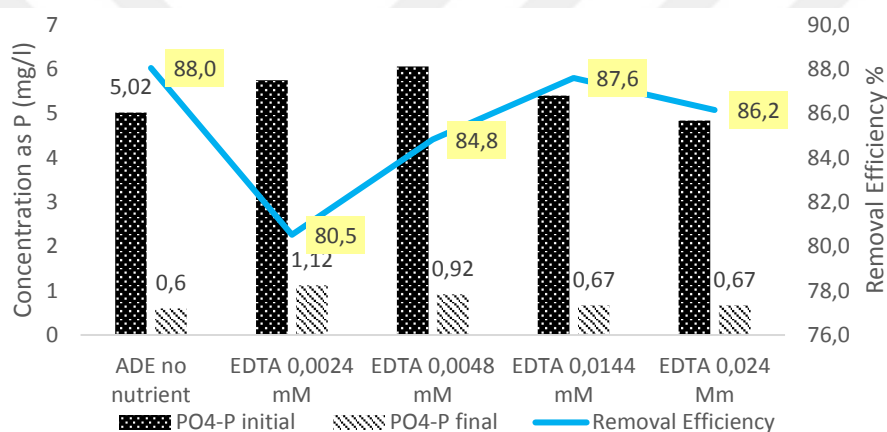


Figure 4.14 : PO₄-P removal of the EDTA solutions.

4.4.1 Solutions containing Mg source and EDTA.

Mg source and EDTA were added to the digestate media in different concentration each. Their concentration values were given in Table 4.11. Comparing solutions Mg 0.6+EDTA 0.0048 and Mg 0.6+EDTA 0.024, growth supported when EDTA concentration was increased. Mg 0.6+EDTA 0.024 solution had better values than Mg 0.6+EDTA 0.0048 in terms of growth. When solutions Mg 2.4+EDTA 0.0048 mM and Mg 2.4+EDTA 0.024mM compared, increased EDTA concentration supported growth more than Mg. Mg 0.6+EDTA 0.024 had the highest biomass productivity, growth rate and doubling time as seen in Table 4.11.

Mg 2.4+EDTA 0.024 and Mg 2.4+EDTA 0.0048 had the highest chlorophyll concentration compared to Mg 0.6+EDTA 0.0048 and Mg 0.6+EDTA 0.024 as expected (Figure 4.15). Because Mg was main component of the chlorophyll pigment (in Figure 2.3). Therefore, chlorophyll concentration was expected to increase as Mg concentration increased. But Mg source concentration as 2.4 mM had similar chlorophyll values with the digestate media. Mg source concentration as 0.6 mM had lower chlorophyll values than the digestate. At this point light penetration effect could be considered. Because, the production of chlorophyll was not only affected to the present of the Mg, it was also affected with light density. Briefly, when there was no enough light, Mg would not be utilized to produce chlorophyll.

Table 4.11 : The biomass concentration, growth rate and generation time.

mM	VSS _{initial} (mg/l)	VSS _{final} (mg/l)	VSS _{net} (mg/l)	μ (d ⁻¹)	TD (d)
Digestate	455	1150	695	0.0982	7.0
Mg2.4+EDTA 0.024	405	1100	695	0.1447	4.7
Mg2.4+EDTA 0.0048	400	1100	700	0.1259	5.5
Mg0.6+EDTA 0.0048	450	1100	650	0.1298	5.3
Mg0.6+EDTA 0.024	450	1180	730	0.1668	4.1

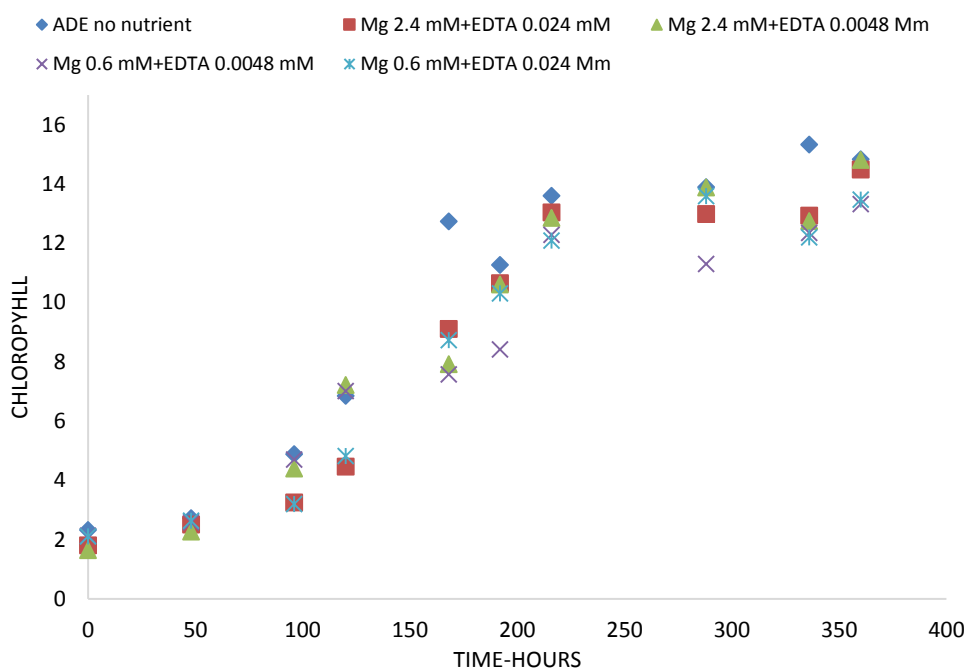


Figure 4.15 : Chlorophyll concentration of the EDTA+Mg.

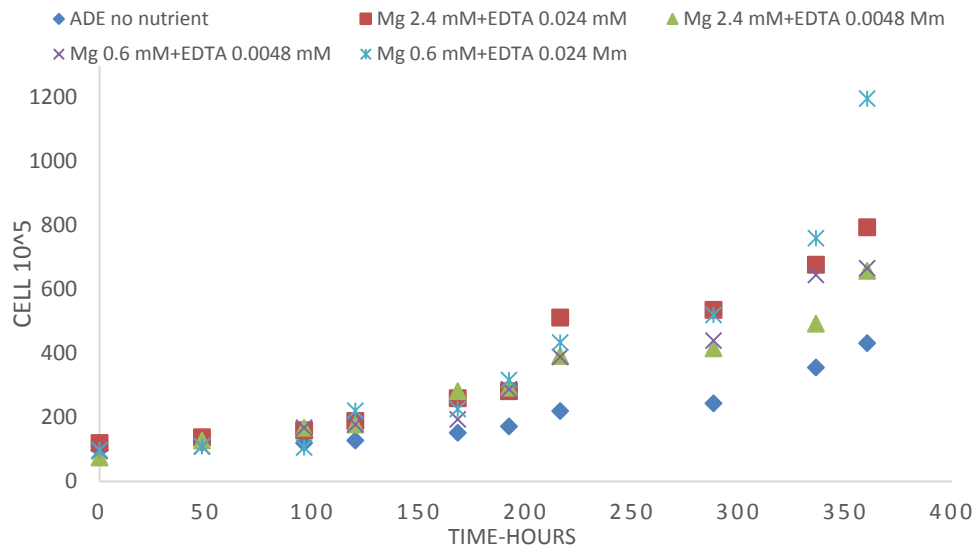


Figure 4.16 : Cell numbers of the EDTA+Mg.

In all solutions, experimental biomass were lower than the theoretical biomass concentration (Table 4.11). 3.6, 6.6, 3.6, 4.6 and 6.66 mg/l/d were calculated nitrogen removal rate for the digestate, Mg2.4 +EDTA 0.024 mM, Mg2.4 +EDTA 0.0048 mM, Mg 0.6 +EDTA 0.0048 mM and Mg 0.6 +EDTA 0.024 mM respectively. Mg 2.4 +EDTA 0.024 mM and Mg 0.6 +EDTA 0.024 mM had higher nitrogen removal rate than others. Therefore, it could be said that 0.024 EDTA mM had a positive effect on nitrogen removal rate.

Table 4.12 : Comparision the theoretical and experimental biomass based on TKN.

mM	TKN _{ini}	Concentration mg/L				
		NH ₃ -N free	Net TKN	Consumed	Theoretical VSS _{algae}	Experimental VSS _{algae}
Digesrate	416	7.4	409	54	861	695
Mg 2.4+EDTA 0.024	442	7.9	434	99	1570	695
Mg 2.4+EDTA 0.0048	447	8.0	439	55	872	700
Mg 0.6+EDTA 0.0048	445	7.9	437	70	1110	650
Mg 0.6+EDTA 0.024	460	8.2	451	100	1597	730

Table 4.13 : Comparision the theoretical and experimental biomass based on NH₃-N.

Unit (mg/L)→ Solutions mM↓	Consumed NH ₄ -N	Theoretical VSS	Experimental VSS
Mg 2.4+EDTA 0.024	44.8	710	695
Mg 2.4+EDTA 0.0048	47.8	757	695
Mg 0.6+EDTA 0.0048	55.0	873	700
Mg 0.6+EDTA 0.024	50.0	792	650
Mg 2.4+EDTA 0.024	55.0	872	730

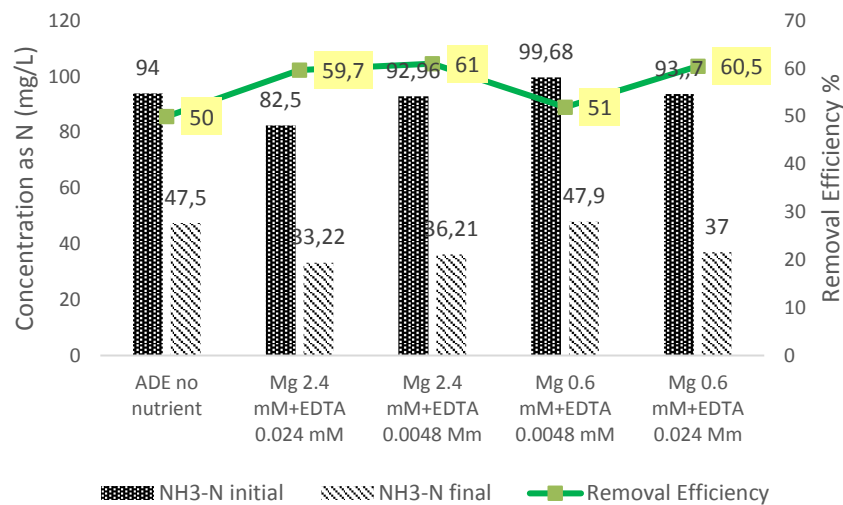


Figure 4.17 : $\text{NH}_3\text{-N}$ removal of the EDTA+Mg.

When COD removals in Figure 4.18 considered, solutions containing Mg2.4 +EDTA 0.024 mM had the highest value. But the others showed similar results like solutions containing EDTA and Mg separately. At this point the precision of the COD removal data of Mg2.4 +EDTA 0.024 was controversial. Phosphorus removals of the solutions were not significant as seen in Figure 20 as EDTA results.

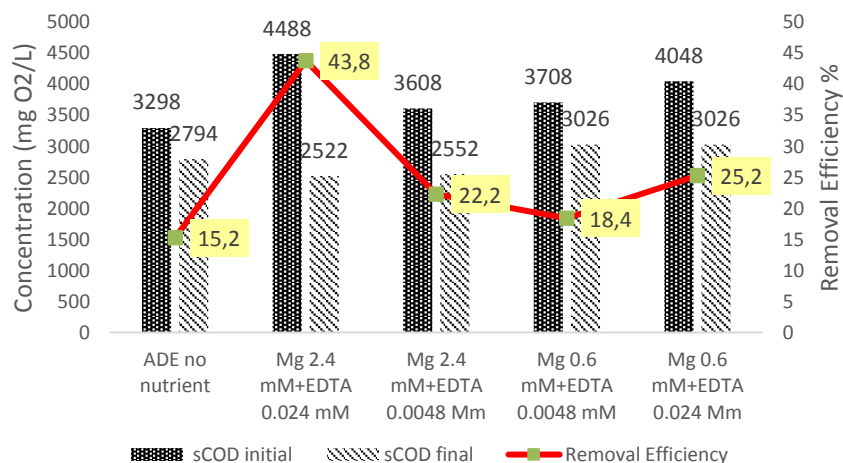


Figure 4.18 : COD removal of the EDTA +Mg.

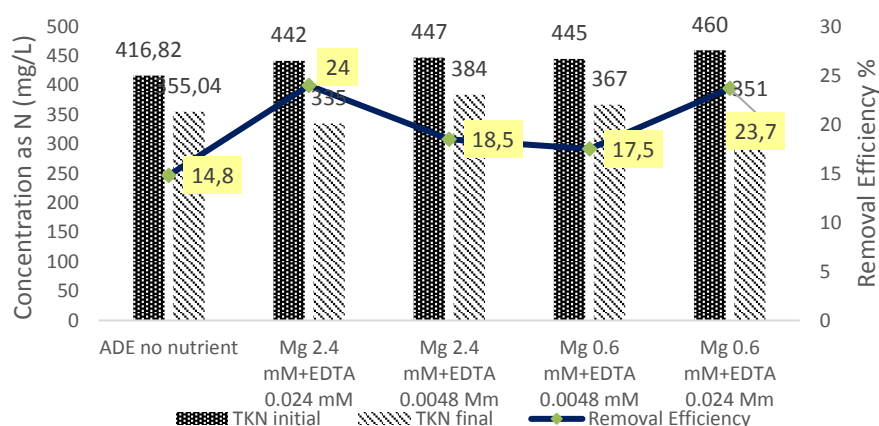


Figure 4.19 : TKN removal efficiencies of the EDTA+Mg.

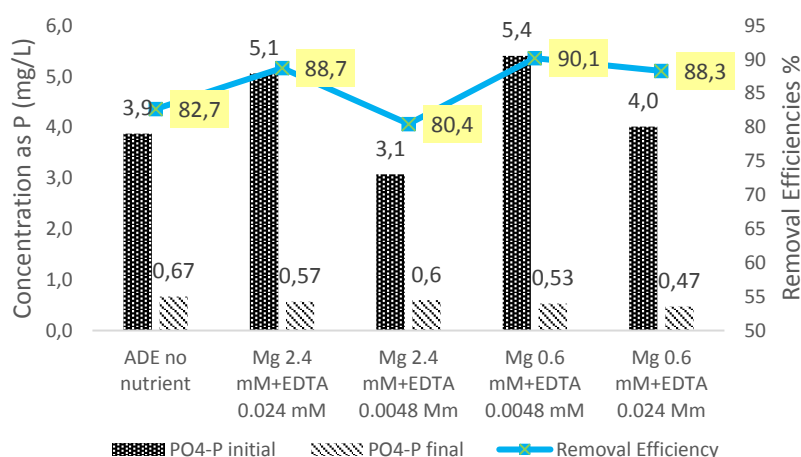


Figure 4.20 : PO₄³⁻-P removal efficiencies of the EDTA+Mg.

4.5 Iron

Iron source was ferric ammonium citrate and it was added to digestate solutions in different concentrations like 0.3; 0.6; 1.2; 2.4 and 3.0 mM as seen in Table 4.14. Among the solutions, when Fe source added into solution with the concentration increased to 10 times as 3.0 mM, inhibition was observed in growth. This inhibition was also seen in chlorophyll concentration and cell numbers in Figure 4.21 and 4.22 respectively.

Although 1.2. mM Fe concentration supported growth, its effect on removal efficiency of the PO₄-P and NH₃-N were very low compared to digestate solution, Mg and EDTA (Table 4.15). Among the Fe solutions, the best result was seen in 1.2 mM Fe concentration with net 920 mg/L VSS with 0.1608 μ (d⁻¹) and 4.3 day doubling time.

7.06, 6.5, 9.13, 5.0, 8.2 and 2.9 mg/l/d were calculated nitrogen removal rate for the digestate, 0.3, 0.6, 1.2, 2.4 and 3.0 mM Fe respectively. Theoretical biomass of the Fe solutions were lower than experimental biomass data like other solutions (Table 4.12). And all experiments supported the idea that 3.0 mM Fe source caused inhibition on growth.

Table 4.14 : The biomass concentration, growth rate and generation time of the Fe.

mM	VSS _{initial} (mg/l)	VSS _{final} (mg/l)	VSS _{net} (mg/l)	μ (d ⁻¹)	TD (d)
Digestate (1:10)	410	1200	790	0.1331	5.2
Fe 0.3 mM	450	1340	890	0.0978	7.0
Fe 0.6 mM	490	1280	790	0.1306	5.3
Fe 1.2 mM	460	1380	920	0.1608	4.3
Fe 2.4 mM	450	1160	710	0.1108	6.2
Fe 3.0 mM	360	1020	660	0.0816	8.4

Table 4.15 : Theoretical and experimental biomass comparison of the Fe based on TKN.

Concentration mg/L						
mM	TKN _{ini}	NH ₃ -N free	Net TKN	Consumed	Theoretical VSS _{algae}	Experimental VSS algae
Digesrate	557	10	547	106	1692	790
Fe 0.3	617	11	606	98	1563	890
Fe 0.6	674	12	662	137	2183	790
Fe 1.2	517	9.2	507	75	1199	930
Fe 2.4	667	11.9	655	123	1959	710
Fe 3.0	598	10.7	587	44	703	660

Table 4.16 : Theoretical and experimental biomass comparison of the Fe based on NH₃-N.

Unit (mg/L)→ Solutions↓	Consumed NH ₄ -N	Theoretical VSS	Experimental VSS
Digesrate	44.8	710	790
Fe 0,3 mM	42.3	671	890
Fe 0,6 mM	41.0	650	790
Fe 1,2 mM	53.5	848	930
Fe 2,4 mM	43.1	684	710
Fe 3 mM	26.7	423	660

Table 4.17 : The removals of the Fe solutions.

	Removal Efficiency %	
	PO ₄ ³⁻ -P	NH ₃ -N
Digestate	88	62
Fe 0.3	62	47
Fe 0.6	45	45
Fe 1.2	48	58
Fe 2.4	58	48
Fe 3.0	60	30

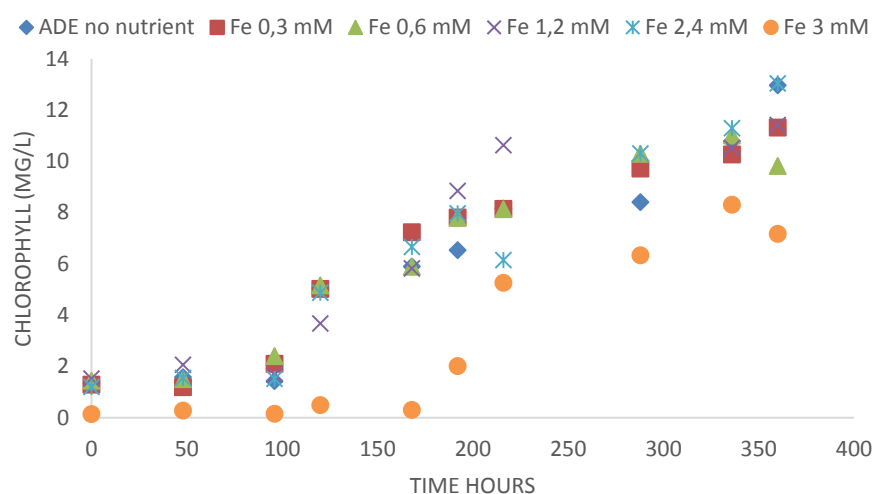


Figure 4.21 : Chlorophyll concentration of the Fe solutions.

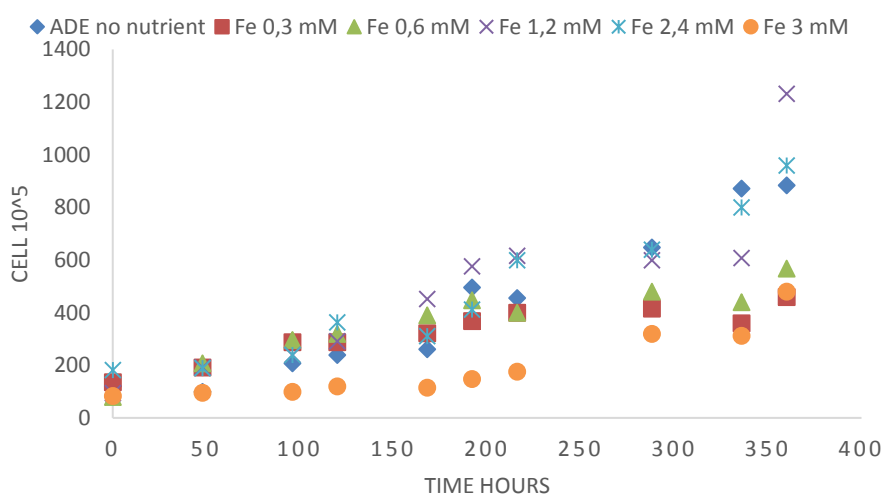


Figure 4.22 : Cell numbers of the Fe solutions.

4.6 Comparison Theoretical and Experimental Biomass for All Solutions

Theoretical biomass of all solutions were higher than experimental biomass in terms of soluble TKN values. Consumed soluble TKN showed how much TKN converted into $\text{NH}_4\text{-N}$. Based on TKN conversion the biomass productivity must have been high. When $\text{NH}_3\text{-N}$ utilization calculated rather than TKN and consumed $\text{NH}_4\text{-N}$ values converted into theoretical biomass, the theoretical biomass values were similar to experimental biomass as it was seen in Table 4.6, Table 4.9, Table 4.14 and Table 4.16 for all solutions.

The digestate media did not only contain microalgae, it had also bacteria and protozoa. Therefore, the low biomass productivity compared to theoretical biomass could be explained as another microorganisms like bacteria and protozoa consumed $\text{NH}_4\text{-N}$ in terms of TKN.

In addition, COD and TKN removals were too lower compared to ammonical nitrogen and phosphorus removals. These low COD removals could have done by bacteria and protozoa in the digestate. If high COD removals obtained related with increased biomass, it would be considered that there was mixotrophic growth in the media. Based on the results of the removals, it was considered that there was probably no mixotrophic growth. In addition, the digestate had to contain glucose and acetate as C source to support mixotrophic growth as it was mentioned in detail literature section. In the digestate, there was no glucose and acetate because they had already been used by microorganisms in the biogas production processes.

4.7 Morphological Characterization of Mixed Microalgal Species

In this study mixed microalgae culture was used. *Chlorella sp.* and *Scenedesmus sp.* were observed as dominant species as they are seen in Figure 4.23. D and E were showing *Scenedesmus sp.*; and *Chlorella sp.* was clearly seen in C. In addition, some of them could not be identified seen in B and F in the picture. The formation of colonization like in B was generally seen in digestate with EDTA. During the experiment, there were also some motile protozoa with cilia and flagella in the media. Taking a picture of protozoa was hard because they were all transparent in the digestate solution and motile. A motile protozoa with cilia captured in the G picture. These protozoa came probably from tap water that was used for the dilution of the digestate.

Because when digestate itself observed in the light microscope, no motile protozoa was observed. In addition it was known that the digestate itself had its own bacteria culture and it was hard to observe these microorganism without staining by using the light microscope. Bacteria and these motile protozoa had probably caused the TKN and COD removals rather than microalgae culture.

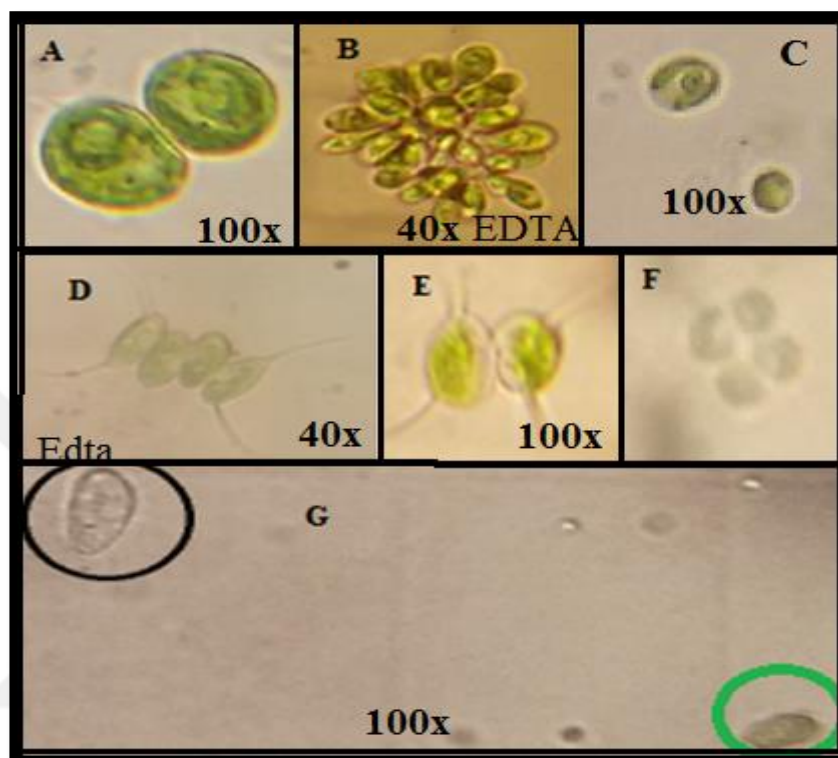


Figure 4.23 : Pictures of the Microalgae sp in the light microscope A is chlorella sp; B and C showing colonies, D and E scenesmus sp, F shows microalgae tetra shaped, G black circle shows a protozoa with silica , yellow circle shows another microalgae.

5. CONCLUSIONS AND RECOMMENDATIONS

This study showed that microalgae grew in the digestate and additional nutrients had an impact on growth; but the support of the growth increased when addition nutrient concentration increased. Therefore more optimization studies were needed as recommendation.

Mg was expected to support growth well. But Mg solutions' results showed lower values compared to EDTA. Among the Mg solutions, only 3.0 mM Mg increased the growth rate and doubling time like $0.1585 \mu^{-1}$ and 4.3 day respectively. When Mg and EDTA added to solutions in different concentrations, Mg 0.6 +EDTA 0.024 had the highest growth rate and doubling time like $0.1668 \mu^{-1}$ and 4.1.day respectively.

As for Fe source, 0.3 mM did not make a significant difference like EDTA 0.0024mM. But when its concentration increased to 10 times as 3.0mM, inhibition was observed in growth. The best result was seen in 1.2 mM Fe source concentration. Among the Fe solutions, the best result was seen in 1.2 mM Fe concentration with net 920 mg/L netVSS_{algae} with $0.1608 \mu (d^{-1})$ and 4.3-day doubling time. Different Fe sources should be tried to optimize growth conditions.

EDTA was known as a chelator in the media for microalgae and was intended to ensure metal ions remain dissolved in the media to be taken into by microalgae. 0.0048 mM EDTA did not make a significant effect on growth, but increased 6 times EDTA concentration as 0.0144; and 10 times as 0.024mM, supported growth. 0.024mM EDTA showed better results compared to the digestate and other solutions in terms of growth and removals. Therefore, it could be said that EDTA had a positive effect on supporting growth. Digestate solution with 0,024mM EDTA had the highest biomass concentration with the value 1410 mg/l VSS_{algae}. with $0.1700 \mu (d^{-1})$ and 4 days doubling time. It had also the highest net VSS value as 1000 mg/L. The best nitrogen removal efficiency showed in EDTA 0.024 mM as 77% following the ammonical nitrogen removal rate with 11.4 mg/l/d and. For a better optimization, different EDTA concentrations with or without nutrients should be completed.

In this study, *Chlorella sp.* and *Scenedesmus sp.* were observed as dominant species via light microscope. Species genetical identification would give more precision idea about species.

As a result, this study showed that microalgae grew in the digestate and additional nutrients had an impact on growth; but the growth rate and doubling time of microalgae cultivated in the digestate media were lower than microalgae cultivated in BG11 media. Therefore, more optimization studies were recommended.



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