Growth optimization of *Acutodesmus obliquus* in anaerobic digested domestic wastewater: advantages and limitations

Dissertation

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Abbreviation

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AGPase	ADP-Glucose Pyrophosphorylase
ADP	Adenosine Diphosphate
AnMBR	Anaerobic Membran Bio Reactor
AOX	Absorbable Organic Xenobiotic
BW	Black Water, wastewater that is originated from toilet waste
BIQ	Bio Intelligence Quotient, an algae house in Wilhemsburg, Hamburg, Germany
BIQ wastewater	wastewater which is obtained from the BIQ building, a mixture of black water (BW) and gray water (GW)
COD	Chemical Oxygen Demand
CDM	Clean Development Mechanism
DOC	Dissolved Organic Carbon
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
EDDHA	Ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid)
EPA	Environmental Protection Agency
Fv	variable Fluorescence
Fm	maximal Fluorescence
GW	Gray Water, wastewater that is originated from washing and shower waste
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
MgAC	Magnesium Aminoclay
$NADP^+$	oxidized form of Nicotinamide Adenine Dinucleotide Phosphate
NO_3-N	Nitrate Nitrogen
NH_4-N	Ammonium Nitrogen
PAM	Pulse Amplitude Modulation
PO_4-P	Phosphorus in a phosphate form
P_{680}	primary electron donor of photosystem II, special form of Chlorophyll a
TAG	Triacylglycerol
TEM	Transmission Electrone Microscopy
TN	Total Nitrogen
TP	Total Phosphorus

TC	Total Carbon
TOC	Total Organic Carbon
UASB	Upflow Anaerobic Sludge Blanket, referred to as a reactor that is used for anaerobic digestion of wastewater
XOC	Xenobiotic Organic Compounds
WW	wastewater

Zusammenfassung

Seit jeher produziert der Mensch Abwasser. Probleme entstehen, wenn die Bevölkerung zunimmt und es an Verarbeitungssystemen mangelt, insbesondere in dicht besiedelten Gebieten. Zu den Umweltauswirkungen gehören u.a. Grundwasserverunreinigungen mit Krankheitserregern und in Folge Ausbreitung von Krankheitsausbrüchen. Eine neue Perspektive ist die Nutzung häusliches Abwasser als potenzielle Energie-, Nährstoff und Wasserquelle (Kujawa-Roeleveld and Zeeman 2006; Zeeman et al. 2008).

Verschiedene Techniken zur Abwasserreinigung wurden eingeführt, etwa physikalische, chemische, biologische oder eine Kombination aus den Genannten. Seit Methangas, das durch einen anaeroben Vergärungsprozess entsteht-, und im Rahmen des Clean Development Mechanism (CDM) des Kyoto-Protokolls im Jahr 1997 als Kohlenstoff-Kredit anerkannt wird, gilt biologische Abwasserreinigung als vielversprechende Technologie.

Die Reinigung häuslichen Abwassers mit Mikroalgen ist nicht neu. Allerdings wurden durch den Einsatz von Mikroalgen in der Abwasserreinigung zahlreiche Vorteile in Bezug auf die Umweltqualität deutlich, wie die Kohlendioxidreduktion, Energieproduktion und effiziente Nährstoffentfernung. Andererseits erzeugt die anaerobe Vergärung von Abwasser Biogas, konserviert Nährstoffe und reduziert Krankheitserreger. Daher ist ein Einbau einer anaeroben Abwasserbehandlung mit anschließender Algennutzung als Nachbehandlung in einem Abwasserbehandlungssystem vorteilhaft.

In einigen Gebieten wurde eine Quellentrennung von häuslichem Abwasser in Grau- (Wasch- und Duschwasser) und Schwarzwasser (Toilettenabwasser) eingeführt. Wegen begrenzter räumlicher Verfügbarkeit wird mancherorts noch immer ein Gemisch dieser häuslichen Abwässer mit einem Abwasserreinigungssystem behandelt. In dieser Arbeit wurde häusliches Abwasser aus 2 unterschiedlichen Standorten untersucht: Zum einen aus einer Siedlung in Lübeck-Flintenbreite (Deutschland), wo Schwarzwasser (BW) von Grauwasser getrennt aufgefangen wird und zum anderen aus dem „Bio Intelligence Quotient“ (BIQ) Wohnhaus in Hamburg-Wilhelmsburg (Deutschland), wo das Abwasser als Gemisch vorlag und deshalb im Folgenden mit „BIQ wastewater“ bezeichnet wird.

In der vorliegenden Arbeit wurden Kultvierungsversuche der Grünalge *Acutodesmus obliquus* in häuslichem Abwasser im Labor- und Feld versuch durchgeführt. Dabei wurden Wachstumslimitierungen, physiologische Leistungsfähigkeit und Nährstoffaufnahmen betrachtet.

Im ersten Teil dieser Arbeit wurde ein optimales Wachstum von *A. obliquus* mit einer Schwarzwasser-Konzentration von 34,33% nachgewiesen. Zudem wurden Wachstumslimitierungen durch die Nährstoffe Magnesium (Mg), Eisen (Fe) und Mangan (Mn) beobachtet, auch bei der Kultivierung in BIQ-Abwasser. Untersuchungen ergaben, dass ein

Wachstum von *A.obliquus* in BW oder in BIQ-Abwasser ab einer Nährstoffkonzentration größer als 0,087-0,114 mg L^{-1} für Mangan und von 0,041-0,079 mg L^{-1} für Eisen stattfindet. Außerdem wurde aus dem BW-Versuch eine Konzentrationsschwelle für Magnesium für das Wachstum beobachtet (1,226 mg L^{-1}). Die Zugabe dieser Wachstum begrenzenden Elemente führte zu einer Zunahme des Wachstums in BW-Abwasser auf 0,357 g Trockengewicht (TG) L^{-1} d^{-1} und im BIQ-Abwasser auf 0,213 g TG L^{-1} d^{-1}. Im Vergleich zum Kontrollmedium ergaben die Untersuchungen mit den Abwassermedien einen 1,2-mal bzw. 1,14-mal höheren Ertrag. Höhere Produktivität von *A. obliquus* in BW wurde von einer höheren Abnahmerate des Gesamt-Sticktoffs (TN) von 80,05% begleitet. Die Reduktion des TN lag bei der Kultivierung im BIQ-Abwasser bei 67,4%, was über der TN-Abnahmerate des Kontrollmediums lag. Dagegen lag die Abnahmerate des Gesamtphosphors (TP) für BW bei 53,15% und im Kontrollmedium bei 59,68%. In den Versuchen mit BW war die Reduktion des TP geringer als bei der Kontrolle, jedoch wurde eine höhere Kohlenstoffakkumulation in den Zellen gezeigt.

Die Bestimmung der Pigmente in der *A.obliquus*-Biomasse ergaben ähnliche Pigmentzusammensetzungen, sowohl bei der Kultivierung im BW als auch im BIQ-Abwasser. Neben Chlorophyll a und b wurden 3 verschiedene Carotinoide als Neoxanthin, Violaxanthin und Lutein identifiziert. Zudem wurde bei der Kultivierung in beiden Abwassermedien eine Chlorophyll-a-Verringerung bei der höchsten Produktivität von *A.obliquus* beobachtet. Dies deutet auf eine Änderung der Ernährungsform von Phototroph zu Photoheterotroph hin, gefolgt von einer Zunahme von Lipiden und Stärke in den Zellen, die mit Hilfe von transmissionselektronenmikroskopischen Untersuchungen von *A.obliquus* bei der Kultivierung in BW beobachtet wurde. Weiterhin bestätigte ein Experiment über den trophischen Modus, dass eine photoheterotrophe Ernährung vorlag. In diesem Experiment hat organischer Kohlenstoff aus dem BIQ-Abwasser das Wachstum von *A.obliquus* gefördert, wenngleich die höchste Produktivität im mixotrophen Modus beobachtet wurde. Nach einer Kultivierungszeit von 5 Tagen ohne anorganischen Kohlenstoffversorgung, wechselte die Ernährungsweise während der exponentiellen Wachstumsphase von der phototrophen zur photoheterotrophen Form. Außerdem zeigte das relativ stabile Verhältnis von Sauerstoffentwicklung zu Atmungsrate von 1,1-2,3 in den BIQ-Abwassermedien ohne anorganische Kohlenstoffzufuhr nach 5 Tagen ein Gleichgewicht im Gasaustauschmetabolismus bzw. zwischen Photosynthese und Atmungsprozess. Dieser Metabolismus hat den Vorteil, dass die künstliche Zufuhr von anorganischem Kohlenstoff reduziert werden kann und somit die Betriebskosten der Algenkultivierung verringert werden.

In einem der wiederholten Batch-Versuche wurde eine Abnahme der Produktivität von 0,65 auf 0,199 g L-1 d-1 beobachtet, trotz Zugabe der Elemente Mn, Mg und Fe. Da außerdem keine weiteren Wachstumsbegrenzungen festgestellt wurden, weist dieses Ergebnis auf das Vorhandensein inhibierender Substanz(en) im Abwasser hin. Eine weitere Batch-Untersuchung mit dem Abwasser zeigte wiederum eine klare Wachstumsbegrenzung durch Mn, Mg und Fe

und die Zunahme der Wachstumsrate von 0,258 auf 0,4 g L-1 d-1 nach der Zugabe dieser drei Elemente. Zudem wurden TN und TP zu über 94% aus dem Abwasser entfernt. Das Ziel eine hohe Biomasseproduktion aufrechtzuerhalten und die Kultivierungsdauer zu reduzieren ist möglich, wenn man diese Ergebnisse beachtet.

Die Kultivierung von *A.obliquus* in anaerob fermentierten BIQ-Abwässern im Freiland Maßstab zeigte keine Lichtlimitierungen. Im Starklicht wurde die höchste Wachstumsrate von 0,29 g L^{-1} d^{-1} beobachtet, dies entsprach etwa 86,83% bei Schwachlicht. Diese Ergebnisse liegen im Bereich anderer Untersuchungen mit BIQ-Abwasser (0,03-0,65 g L^{-1} d^{-1}). Diese Ergebnisse sind vielversprechend, da im Vergleich zum Labormaßstab in einer ähnlichen Kultivierungszeit viel mehr Biomasse von einer Photobioreaktor-Außenfassade produziert werden könnte.

Summary

Domestic wastewater has been always produced throughout human history. Problems arise as the size of populations increase without appropriate wastewater treatment facilities, especially in densely populated areas. Among the environmental impacts are groundwater contamination with pathogens and the outbreak and spreading of diseases. However, a new perspective considers domestic wastewater as potential resources for energy, nutrients and water (Kujawa-Roeleveld and Zeeman 2006; Zeeman et al. 2008).

Various techniques in wastewater treatments have been introduced, including physical, chemical and biological methods used alone or in combination. Methane gas generated by the process of anaerobic digestion was approved as a carbon credit in the Clean Development Mechanism (CDM) under the Kyoto Protocol in 1997 and since that time, biological wastewater treatment has been recognized as a promising technology (Chan et al. 2009).

Domestic wastewater treatment using microalgal culture is not new. However, by applying algae in wastewater treatment, various advantages and improvements have been observed, such as a reduction in carbon dioxide emission, an increase in energy production and more efficient removal of nutrients. In addition, valuable biomass would be another benefit which meets the economic criteria in such wastewater treatment operation with algae. On the other hand, anaerobic digestion of wastewater is recognized for generation of biogas, preserves nutrients and reduces pathogens. Therefore, an incorporation of anaerobic wastewater treatment with algae utilization as a post-treatment in a wastewater treatment system will initiate various advantages.

In some areas, source separation of domestic wastewater in gray (washing and shower waste) and black (toilet waste) water has been introduced. On the other hand, due to space limitations, a mixture of domestic wastewater in a wastewater treatment system is still operated in some places.

In this study, domestic wastewater was collected from 2 places: a source separated black water (BW) from a human settlement in Lübeck-Flintenbreite, Germany and a mixture of black and gray water from an apartment building, called Bio Intelligence Quotient (BIQ), in Wilhemsburg, Hamburg, Germany. Laboratory and up-scaled outdoor experiments were carried out in this study to observe growth limitations, physiological performance and nutrients removal by the green alga *Acutodesmus obliquus* in domestic wastewater.

In the first part of this study, a BW concentration of 34.33% was found to be optimal to support the growth of *A. obliquus*. However, growth limitation was observed by magnesium (Mg), iron (Fe) and manganese (Mn). Such elemental limitation was also examined in *A. obliquus* cultivation with BIQ wastewater. Growth limitation of *A. obliquus* in BW or in BIQ wastewater occurred in the range of 0.087-0.114 mg L^{-1} for Mn and 0.042-0.082 mg L^{-1} for Fe. In addition, growth limitation of Mg from the BW experiment was 1.226 mg L^{-1}. Addition of these

limited elements resulted in a productivity in BW media of 0.357 g L^{-1} d^{-1}, which was 1.2 times higher than that in artificial culture medium. In the experiment with BIQ wastewater, which was added with limited elements, lower productivity (0.213 g L^{-1} d^{-1}) was achieved than in BW. However, this productivity value was 1.14 times higher than in artificial medium. Higher productivity of *A. obliquus* in BW was accompanied by higher total nitrogen (TN) removal of 80.05%, compared to 67.4% in BIQ wastewater. Nevertheless, such percentages of TN removal in the BIQ wastewater were higher than in the control medium. Contrary, total phosphorus (TP) was removed to a lower extent of 53.15% in BW than in the control medium of 59.68%. In BW, the lower TP removal than in control was accompanied by a higher carbon accumulation in the cells.

Similar pigments were produced by *A. obliquus*, both cultivated in BW and in the BIQ wastewater. Besides chlorophyll a and b, 3 different carotenoids were identified as neoxanthin, violaxanthin and lutein. Nevertheless, in both wastewater media, chlorophyll a reduction was observed with the highest productivity of *A. obliquus*. This indicates a change of the trophic mode from phototrophic to photoheterotrophic, which was followed by accumulation of lipids and starch within the cells in BW medium. This phenomenon was observed by transmission electron microphotograph of *A. obliquus* cultivated in BW. The indication of the photoheterotrophic nutrition was strengthened by the results from a trophic mode experiment. In this experiment, organic carbon of BIQ wastewater was able to support growth of *A. obliquus*, although highest productivity was observed in the mixotrophic mode. After a long adaptation of 5 days in the culture without inorganic carbon supply, the nutrition switched from the phototrophic to photoheterotrophic mode during the exponential growth phase. In addition, the relative stable oxygen evolution to respiration rate ratio of 1.1-2.3 in the BIQ wastewater media without inorganic carbon supply after day-5 indicated a balance in the gas exchange metabolism or between photosynthesis and the respiration process. This metabolism has an advantage since artificial inorganic carbon supply can be reduced and thus, reduces operational cost of alga cultivation.

In one of the repeated batch experiment, a decrease of productivity from 0.65 to 0.199 g L^{-1} d^{-1} was observed. At first, it was assumed that growth was limited by other micro elements than Mn, Mg and Fe. But besides Mn, Mg and Fe, growth limitation by other elements was not determined. This indicated the presence of an interfering substance. However, another repeated batch experiment showed more consistent results with the first batch experiment with the BIQ wastewater, since growth limitation by Mg, Mn and Fe was observed. In this experiment, productivity increased from 0.258 to 0.4 g L^{-1} d^{-1} and nutrients of TN and TP were removed above 94%. The purpose of maintaining high biomass production and reduction of the cultivation period is possible by considering these results.

Cultivation of *A. obliquus* with anaerobic digested BIQ wastewater in outdoor scale experiment showed no light limitation. The highest productivity of 0.29 g L^{-1} d^{-1} in high light

experiment was only 86.83% of that in low light. However, such productivities were in the range of other BIQ wastewater experiments (0.03-0.65 g L^{-1} d^{-1}). This result is promising, as compared to laboratory scales, much more biomass could be produced by the outdoor photobioreactor façades in a similar cultivation period.

1. Introduction

1.1 Domestic wastewater: nutrients, energy and water resource

Domestic wastewater is an unlimited resource of nutrients, water, and energy. Urbanization in developing countries, a demographic trend in the 21st century, is particularly associated with rapid population growth (Parkinson and Tayler 2003). Urbanization hence generates a significant increase in population as well as domestic waste, both liquid and solid. These conditions could cause other problems, especially if such waste is disposed without treatment. Ground water, as well as surface water contamination, may arise. Eutrophication is one water contamination problem that has already affected lakes, reservoirs, estuaries, and coastal waters, especially in populous countries (Bouwman et al. 2005). This is not found only in developing countries; an outbreak of cholera occurred in Hamburg, Germany in 1892 due to contamination of water resources (Otterpohl et al., 2002).

As mentioned by Mara (2004) and the Environmental Protection Agency (EPA) (2017), domestic wastewater is defined as the wastewater generated from human body excrements as well as the water used for toilet flushing, personal washing, laundry, and kitchen activities (i.e. household activities).

Based on the idea of innovative technology in the wastewater management process, source separation of household wastewater has been introduced (Otterpohl et al. 2002). In this concept, household wastewater is differentiated into black water for toilet waste and gray water for washing and shower waste. Both types of wastewater have the potential to be utilized for nutrients and energy resources (black water) and water, nutrients, and energy resources (gray water) (Zeeman et al. 2008). The characteristics of black water and gray water varies in different area, depending on food intake and water/ chemical usage (Kujawa-Roeleveld and Zeeman 2006). Referring to Zeeman et al. (2008), the lowest volume within black water produced by a single person per day contains 6.8 g of total nitrogen (TN) and 0.68 g of total phosphorus (TP), and the lowest volume within gray water contains 0.48 g of TN and 0.3 g of TP (Table 1). On the other hand, Kujawa-Roeleveld and Zeeman (2006) reported that among the domestic wastewater, more than 60% of TN and TP originated from black water, whereas 9% of TN and 20% of TP came from gray water (Table 1). Hence, an efficient recovery process is preferable to avoid such valuable resources being lost.

The energy potential of domestic wastewater has been studied for decades. The anaerobic digestion process has become of interest in improving domestic wastewater treatment, particularly since the 1970s, when energy crises first began (Foresti et al. 2006). With different temperature settings, Zeeman et al. (2008) showed 5-14 L d^{-1} of methane (CH_4) production from 6-7 L d^{-1} of black water, in which the highest CH_4 production was generated in

the highest temperature of an Upflow Anaerobic Sludge Blanket (UASB) reactor at 25^0C. Biogas produced from an anaerobic digestion process consists of CH_4 (55-75 vol.%) and carbon dioxide (CO_2) (25-45 vol.%), which can be utilized for electricity cogeneration as well as heating system (de Mes et al. 2003).

Table 1. Potential nutrient resources from black water and gray water

	Volume (l person^{-1}d^{-1}) [(a)]	TN (gl^{-1})[(a)]	TP (gl^{-1})[(a)]	TN (% of all kind of domestic wastewaters)[(b)]	TP (% of all kind of domestic wastewater)[(b)]
Black water	6.8 -7.5	1 - 2	0.1 - 0.3	82	68
Grey water	60 - 90	0.008 – 0.019	0.005 - 0.018	9	20

[(a)](Zeeman et al. 2008)
[(b)](Kujawa-Roeleveld and Zeeman 2006)

Through appropriate application of membrane or reverse-osmosis techniques, as well as source separation between gray and black water, it was found to be possible to reuse wastewater as a valuable fresh water resource (Otterpohl et al. 2002). Hence, wastewater reuse could supply fresh water to areas where water is scarce.

1.2 Wastewater treatment

It is known that wastewater treatment can be performed physically, chemically or biologically, implemented on their own or in combination. Physical treatment is usually used first by using mechanical system to remove solid contaminants, while chemical treatment is designed to remove dissolved pollutants for an improvement of wastewater quality. Chemical treatment is usually used in pairs with physical and biological treatment (Rawat et al. 2010). However, since physical and chemical treatments are costly, and sometimes even change chemical conditions, such as pH and conductivity, biological wastewater treatment has been reported to be better (Renuka et al. 2015). To design a wastewater treatment system, certain wastewater characteristics and kinds of contaminants should be considered. If a certain contaminant removal level is necessary, individual wastewater treatment needs to be combined with further common primary, secondary and tertiary treatments (Rawat et al. 2010). In such primary treatment, wastewater is collected in an immobile vessel. There, solid parts settle at the bottom of the vessel, while lighter parts, such as oil and grease, float at the surface and are mechanically removed. Subsequently, the middle liquid part is channeled to a secondary treatment plant where suspended materials are eliminated. Tertiary treatment, e.g. algae cultivation, is defined as further treatment after primary and secondary treatments (Rawat et al. 2010).

Biological wastewater treatment has been recognized as a promising technology since carbon credits from the Clean Development Mechanism (CDM) under the Kyoto Protocol in

1997 are attained through methane gas production via an anaerobic process (Chan et al. 2009). The advantages of biological wastewater treatment are its low treatment cost and no generation of secondary pollution.

Chan et al. (2009) reported that anaerobic digestion is beneficial compared to aerobic treatment processes as it is suitable for high concentrations of chemical oxygen demand (COD) (over 4000 mg L^{-1}) as well as low concentration of COD (less than 1000 mg L^{-1}). Other advantages are that less energy is required, bioenergy is generated and nutrients are recovered (Chan et al. 2009). The necessity to preserve the resource potential through wastewater treatment selection process is recognized today (Kujawa-Roeleveld and Zeeman 2006). In general, an anaerobic digestion process produces methane and carbon dioxide as its end products (Figure 1). Since methane can be utilized as biogas energy, it is possible to capture carbon dioxide and channel it to an algae cultivation system. As such, a biological wastewater treatment plant coupled with algae cultivation would be of interest.

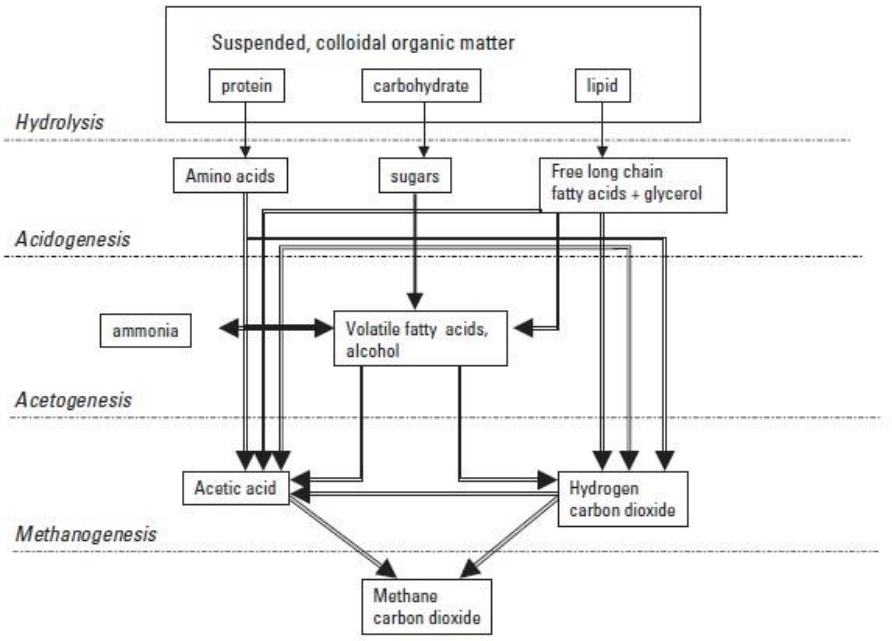


Figure 1. Simplification of an anaerobic digestion process (de Mes et al. 2003)

Domestic waste carries the risk of pathogenic organisms, which could harm people and their surrounding environment. However, in various anaerobic digestion experiments, reduction in pathogens was observed (Salminen and Rintala 2002). To destroy pathogens, Salminen and Rintala (2002) successfully investigated the advantage of using a thermophilic digestion system over a mesophilic one. An anaerobic digestion system, aside from destroying pathogens, preserves most nutrients. However, a disadvantage of such a system is that the effluent will not meet environmental quality standards for environmental discharge due to the high nutrient

concentration. As such, a post-treatment system, such as algae cultivation, in combination with anaerobically digested wastewater, should be beneficial as algal cells are able to remove and accumulate released nutrients.

1.3 Algae in wastewater treatment system

Compared to higher plants, microalgae are more efficient in using solar energy to generate biomass due to their simple cellular structure and easier access to water and nutrients from their aquatic environment (Pires et al. 2013). Various techniques have been proposed to examine the highest productivity efficiency in algal culture. Outdoor mass cultures of algae, e.g. open pond culture systems, and tubular/flat panel photobioreactors were compared (Lee 2001). Both systems have their own advantages and disadvantages. Open pond culture has low capital cost but it is difficult to maintain monoculture conditions. An enclosed photobioreactor is a better solution for maintaining monoculture conditions, with the further advantage of higher productivity (Lee 2001). However, inside the reactor oxygen production and over-heating in summer are factors that need to be considered (Chaumont 1993).

In recent years, microalgae have met the criteria for enhancing environmental quality by means of: 1. reducing carbon dioxide (CO_2) gas emissions by capturing flue gases from industrial activity; 2. producing bioenergy; 3. removing nutrients from wastewater (Pires et al. 2013).

Domestic wastewater treatment using microalgae has been applied for decades (Rawat et al. 2010). Nutrients removal from domestic wastewater has been examined by Tam and Wong (1989) and Wang et al. (2010). In their experiment, Tam and Wong (1989) utilized *Chlorella* and *Scendesmus* to remove nutrients from sewage. They successfully removed two thirds of nitrogen and phosphorous from their wastewater. In their experiment with *Chlorella*, Wang et al. (2010) not only successfully removed N and P, but showed the ability of the system to reduce COD and metal ions (Fe, Mg, Mn, Al and Ca).

The ability of algae to clean non-domestic wastewater is shown in various experiments. With a consortia of filamentous green algae, not only were N and P removed from swine manure, but so were other elements accumulated in the biomass (Kebede-Westhead et al. 2006). In another experiment, with a mixed culture of algae (dominated by *Chlorella* and a diatom species), 58% of chemical oxygen demand (COD), 80% of color and 80% of absorbable organic xenobiotic (AOX) were removed from paper mill wastewater (Tarlan et al. 2002). By utilizing liquid residues from the hydrothermal gasification process, *Acutodesmus obliquus* was able to recover 56.8% of nitrogen and 1.3% of phosphorus (Patzelt et al. 2015a).

Nutrients (nitrogen and phosphorus) recovery from anaerobically treated black water has been examined by Fernandes et al. (2015) without any post-treatment after digestion. In a thirteen-day experiment, the growth of *Chlorella vulgaris*, *C.sorokiana* and *Scenedesmus*

obliquus was observed. These three algal species grew successfully in source-separated anaerobic digested black water and about 4 g L^{-1} cell dry weight was reached. Likewise, 26-30% of ammonium nitrogen (NH_4-N) and 22-93% of phosphorus in phosphate form (PO_4-P) were removed. The batch experiment performed in Erlenmeyer flasks was subsequently continued with another experiment using *C.sorokiana* in 380 ml flat panel photobioreactors, where 100% of phosphorus and nitrogen was removed within 12 days. In another toilet wastewater treatment, *Chlorella sorokiana* was cultivated in non-diluted human urine with the highest growth rate of 0.158 h^{-1} (based on optical density at 750 nm) (Tuantet et al. 2014). In this experiment, growth was not inhibited by ammonium nitrogen (NH_4-N) concentrations up to 1400 mg L^{-1}. About 17% of the cost of commercial algal biomass production is needed for the supply of nutrients (Borowitzka 1992). Hence, usages of such resources make commercial scale algal production economically feasible. Although some algal cultivation experiments using source-separated domestic wastewater, either anaerobically digested or non-digested, have been performed, unclear information remains about growth limitations and stability of such wastewater to support the optimal growth and physiological changes of cells. On the other hand, an anaerobically digested mixture of gray and black water for algae cultivation has never been examined before, particularly in upscaling photobioreactor façades.

1.4 Microalgae: valuable biomass

My hypothesis is that algae cultivation, as a post-treatment system, would work efficiently after anaerobic digestion of wastewater, and this would make it possible to efficiently remove some pollutants from effluent. Hence, algal biomass production will also be a further advantage of such a post-treatment system. More than a hundred thousand species of microalgae may exist. These naturally distributed in marine, freshwater or terrestrial ecosystems (Metting 1996). This represents an enormous diversity of biochemical and metabolic products. Valuable products of microalgae are described in Table 2.

Table 2. Valuable products of microalgae

Application	Products	Algal genus
Human nutrition(1)	Health drink	*Chlorella*
	Antioxidant capsule	*Spirulina*
Animal feed(1)	Feed additive	*Chlorella*
		Spirulina
		Scenedesmus
Aquaculture(1)	Fish feed	*Skeletonema*
		Chaetoceros
		Chlorella

		Spirulina
Biofertilizer[1]	Nitrogen fixation	*Anabaena*
		Nostoc
Valuable substances[1]	Polyunsaturated fatty acid	*Cryptecodinium*
	Antioxidants	*Spirulina*
	Color and food coloring	*Dunaliella*
		Haematococcus
Bioenergy[2],[3]	Hydrogen	*Chlamydomonas reinhardtii*
	Biodiesel	*Botryococcus braunii*
		Chlorella sp.

[1] (Pulz and Gross 2004)
[2] (Ghirardi et al. 2000)
[3] (Chisti 2008)

1.5 Scope of the study

This study was performed as part of the HAWANA project, a research and development project funded by the BMBF (*Bundesministerium für Bildung und Forschung*), in which SSC GmbH cooperated with TU Berlin to develop a technology to interlink decentralized wastewater treatment and photobioreactor façades. Prior to upscaling experiments in outdoor phorobioreactor façades, various laboratory experiments were performed at the Department of Aquatic Ecophysiology and Phycology, Biocenter Klein Flottbek, University of Hamburg. In addition, wastewater treatments were carried out by the Department of Wastewater Management and Water Protection, TUHH (*Technische Universität Hamburg*).

The alga used in this study was *Acutodesmus obliquus*, a green unicellular fresh water alga (Chlorophyceae). This alga has a resistant cell wall (Gruber-Brunhumer et al. 2015), robustness and is proven to grow properly in a large volume of photobioreactors (Patzelt et al. 2015b). Among thirteen freshwater microalgae strains, *A. obliquus* was found to be the most vigorous, the highest productivity, as well as fatty acid and lipid production (Abomohra et al. 2013). In addition, the genus *Acutodesmus* (previously known as *Scenedesmus*) produces high potential carotenoids among the class Chlorophyceae (Paliwal et al. 2016, Grama et al. 2014). Lutein content of 0.53% cell dry weight in *Scenedesmus almeriensis* was higher than the 0.03% observed in a commercial source of lutein compound, marigold flowers (Sánchez et al. 2008). Aside from functioning as a food colorant, lutein is proposed to prevent cancer and diseases related to retinal degeneration. Lutein demand in the world is still high to meet the daily consumption recommendation of 6 mg of lutein per day (Sánchez et al. 2008).

Wastewater used in this study was collected from two sites: 1. a human settlement of 100 inhabitants in a peri-urban area in Lübeck-Flintenbreite, Germany; 2. an apartment building, known as the Bio Intelligence Quotient (BIQ) building in Wilhemsburg, Hamburg, Germany, which consists of 15 residential units of 50-120 m^2 and holds about 30 inhabitants. The

domestic wastewater from Lübeck was source-separated black water, while a mixture of black and gray water was collected from the BIQ building.

In general, this study involved laboratory and up-scaled outdoor experiments. Wastewater treatment was implemented after anaerobic digestion process, subsequent sterile-filtration, and ultra violet (UV) exposition to avoid any risk of pathogenic organisms before algae cultivation. At the laboratory scale, growth of *A. obliquus* in various compositions of domestic wastewater was followed by biomass production, as well as changes in nutrients concentration, and various physiological performances within cells. Further experiments on different trophic modes were carried out, completed with oxygen evolution and respiration rate measurement. Finally, up-scaling experiments in outdoor photobioreactor façades were performed during high and low natural light intensities.

1.6 Objectives

Anaerobically digested black water is known for its potential source of nutrients for microalgae. However, questions arise regarding other factors that may limit the optimal growth of algae in such wastewater, especially for *A. obliquus*, which is a species of special interest. Aside from examining growth limitations, the black water experiment with *A. obliquus* was intended to observe physiological performance, including nutrient uptake by the cells.

A comparative experiment with similar objectives was run using a mixture of black and gray water to observe the growth limitations, physiological performance and nutrient uptake of *A. obliquus*. To continue the first experiment with BIQ wastewater, repeated batch experiments were performed, seeking to examine stable productivity among repeated batch periods. The results of pigment analysis raised the question whether other trophic modes should be performed. Therefore, different trophic mode experiment was carried out to answer following questions: 1. Is *A. obliquus* able to grow in trophic mode other than phototroph in BIQ wastewater? ; 2. Can the organic content in the BIQ wastewater support the growth of *A. obliquus*, i.e. is the organic carbon content an additional C source for the algae?.

Upscaling outdoor experiments in photobioreactor façades were carried out, with the objective of comparing the results of biomass production at laboratory and mass outdoor scales as well as different light intensities. In addition, nutrient removal was observed.

2. Material and Methods

2.1. Algal material used in the study

The algal species used in this study was the green alga *Acutodesmus obliquus* (No. 10169), which was obtained from the Microalgae and Zygnematophyceae Collection in Hamburg (MZCH) (von Schwartzenberg et al. 2013). The algal species was isolated from a microalgae cultivation facility in Reitbrook, Hamburg, Germany. Detailed information regarding the alga is written on MZCH-SVCK n.d. website.

A. obliquus was pre-cultured in a climate room at the Biozentrum Klein Flottbek, University of Hamburg prior to each experiment. The condition within the climate room was adjusted to a controlled light-to-dark period of 14:10 hours and a temperature of 20^0C. *A. obliquus* used in the experiment always grew in the exponential phase and was continuously aerated with 4% vol. of CO_2 in air for about 10-12 days of pre-culture. Cultivation was prepared in a 1L of Erlenmeyer flask containing KC medium (Kessler, E. and Czygan 1970, Table 4) or 2 g L^{-1} of basic fertilizer (Flory Basisdünger 1, Euflor, Germany, Table 3), supplemented with 3.22 g of KNO_3.

Table 3. Composition of Flory Basisdünger 1 medium

Compound	%
Phosphorus pentoxide (P_2O_5)	14
Potassium oxide (K_2O)	38
Magnesium oxide (MgO)	5
Sulphur trioxide (SO_3)	35
Boron (B)	0.02
Copper as chelate of EDTA	0.003
Iron as chelate of EDTA + DTPA	0.2
Manganese as chelate of EDTA	0.04
Molybdenum	0.006
Zink as chelate of EDTA	0.005

Table 4. Composition of KC medium

Compound	g
KNO_3	0.81
NaCl	0.47
$NaH_2PO_4.2H_2O$	0.47
$Na_2HPO_4.12H_2O$	0.36
$MgSO_4.7H_2O$	0.25
$CaCl_2.2H_2O$	0.014
$FeSO_4.7H_2O$	0.006
$MnCl_2.4H_2O$	0.0005
H_3BO_3	0.0005
$ZnSO_4.7H_2O$	0.0002
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.00002
EDTA (Tritiplex III)	0.008
H_2O	1000 ml

2.2. Experimental design and installation

The experiments conducted in this study consisted of two major parts: laboratory- and outdoor-scale experiments. Four experiments were performed at the laboratory scale, which are cultivation of *A. obliquus* in anaerobic digested black water; in an anaerobic digested mixture of black and gray water; in repeated batches; and in different trophic modes. In all laboratory-scale experiments, the same aeration system with gas containing 4% CO_2, a light:dark period of 14:10 hours, a light intensity of 420 µmol photon m^{-2} s^{-1} and a temperature of 20^0C was applied, except for the trophic mode experiment where different gas compositions were implemented. During the experiment pH was controlled with a pH meter (WTW, Germany) and adjusted to 7.5 with the help of 1M NaOH and 10% or 25% of HCl. Outdoor pilot plant-scale experiments consisted of two different experimental conditions: high natural light and low natural light intensities.

2.2.1. Batch cultivation in anaerobic digested black water (BW)

The black water was obtained from a collecting tank at Lübeck-Flintenbreite, Germany. About 10 L of black water was sent to TU Berlin and was anaerobically digested for 21 days. The fermented BW was further centrifuged at 4000 rpm for 10 minutes (Multifuge X3, Thermo Scientific, USA) and the supernatant was gradually filtered with membrane filters with pore sizes of 2.7 µm (Microfiber filters, Whatman, Germany), 1,2 µm (GF/C, Whatman, Germany), 0.45 µm (Whatman, 7404-004, Germany) and 0.1 µm (Schleicher and Schüll PH 79, Germany) under pressure filtration (Figure 2A). Additionally, the supernatant was exposed to ultraviolet C (UV C) for sterilization (Figure 2B) prior to the experiments. These filtration and UV sterilization processes were conducted at TUHH, Hamburg, Germany.

A **B**

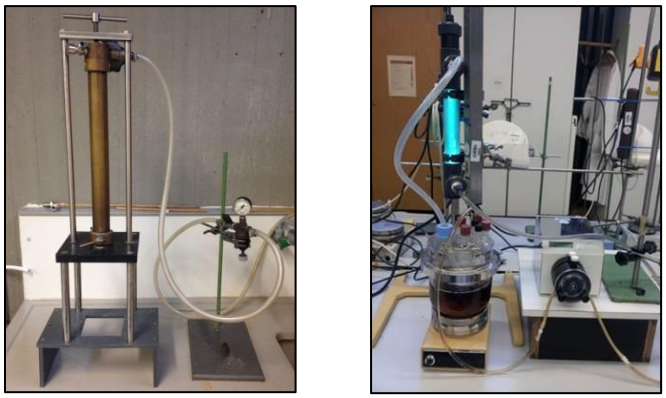


Figure 2. Pressure filtration (A) and UV sterilization process (B)

2.2.1.1. Determination of optimal black water concentration in growth media

The first experiment with anaerobic digested black water was aimed to observe the optimal BW concentration within the culture medium to support optimal growth of *A. obliquus*. This experiment was conducted in a Kniese system, consisting of 12 glass tubes within a temperated water bath, lighting and gas supply systems. The length of each tube was 45 cm and had a maximal working volume of 350 ml.

To determine the range of optimal BW concentrations for supporting the growth of *A. obliquus*, three different BW concentrations were prepared: 34.33%, 11,4% and 5%. Each concentration experiment was performed in triplicate. The highest concentration of 34.33% BW was adjusted based on the nitrogen concentration of the basic fertilizer (446.3 mg N L^{-1}). As a control, Flory Basisdünger 1 (Euflor, Germany) was used with additional KNO_3 of 3.22 g. At day-8, referring to the KC medium (Kessler, E. and Czygan 1970), all elements (except nitrogen- and phosphorus-containing compounds) were added to the cultures in BW. Optical density of algal medium and algal photosynthetic activity were measured every day, while cell dry weight and pH were measured every 5 days.

2.2.1.2. Elemental limitation experiment

Based on elemental analysis of the BW sample, the second experiment was aimed to observe elemental limitation of manganese (Mn), magnesium (Mg) and iron (Fe). Supplements of these elements shall support optimal growth of *A. obliquus*. The experiment was set up in multi-cultivator systems (MC 1000-ODMC 1000-OD, Photon System Instrument, Czech Republic) (Figure 3). One multi-cultivator system consists of eight tubes with a working volume of 80 ml for each tube. Since the experiment was performed in triplicate, two multi-cultivators were used in parallel.

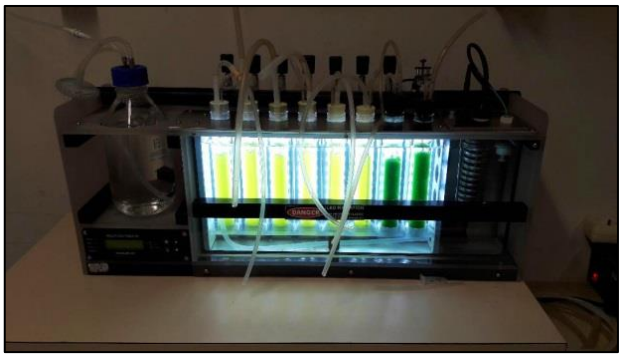


Figure 3. Multicultivator system for alga culture

In this experiment, 3 media compositions of different elements and BW, 1 media of only BW and a control medium of modified KC were set up. Besides 34.33% of BW, the elements added in medium A are: Mn and Mg, in medium B: Mn and Fe and in medium C: Mn, Mg and

Fe. The magnesium, manganese and iron were supplemented referring to the KC medium. The KNO_3 concentration in the modified KC medium, as the control medium, was adjusted to the same nitrogen concentration of 34.33% of BW. The BW was diluted with tap water. Each concentration was performed in triplicate.

In this experiment, more parameters were measured than in the previous BW experiment. Optical density (OD) and photosynthetic activity were measured every day, while cell dry weight was measured in certain periods until stationary growth (observed from OD values) was achieved. Element concentration analysis was carried out by Helmholtz Zentrum Geesthacht (HZG), Zentrum für Material und Küstenforschung, Geesthacht, Germany. The method used for elemental analysis is explained in sub-chapter 2.3.3.4. The solute element concentrations were determined from which elements remained in the media at the stationary phase. Carbon, nitrogen and phosphorus concentrations in cells, as well as those that remained in the media, were measured in the beginning and at the end of experiment. Pigment analysis and transmission electron microphotographs were only performed at the end of experiment.

2.2.2. Batch cultivation in the BIQ wastewater

In this experiment, anaerobic digestion of the BIQ wastewater was performed in a fermenter at the laboratory facility at the algal pilot plant in Reitbrook, Germany. It was carried out in the same manner as the previous experiment with BW. The BIQ wastewater was further centrifuged, filtered up to a filter pore size of 0.1 µm and exposed to UV sterilization prior to experiments.

Based on elemental analysis of the BIQ wastewater, characteristics similar to those of the BW were observed. Manganese, iron, and magnesium concentrations were lower than the KC medium. As such, 3 different BIQ wastewater media compositions were investigated (Medium A: BIQ wastewater with Mn, Mg, and Fe; Medium B: BIQ wastewater with Mn, Mg, Fe, and EDTA; Medium C: pure BIQ wastewater) and KC medium was applied as a control (medium D). Concentrations of supplemented elements referred to the KC medium. The N and P concentration in the control medium was adjusted to those in the BIQ wastewater. In addition, to detect elemental limitation, EDTA was added in one medium to observe the necessity of such a compound for the growth of *A. obliquus*. This experiment was carried out in the Kniese system and each medium composition was performed in triplicate.

To follow the growth kinetics, optical density and cell dry weight were measured and photosynthetic activity was observed using Fv/Fm values. Elemental analysis was performed with media collected at the stationary phase. Nitrogen and phosphorus concentrations were measured in the beginning and at the end of the exponential phase to determine their removal from the media and uptake within cells. Pigment concentrations within cells were analyzed at the end of experiment.

2.2.3. Repeated batch experiments of growth with BIQ wastewater

BIQ wastewater used in this experiment was digested anaerobically in an anaerobic membrane bioreactor (AnMBR) built within the water system of the “BIQ Algen Haus”, Hamburg, Germany. To avoid pathogens, the supernatant was pressed through an ultrafiltration membrane system (Bioflow External Membran Bioreactor, HyperFlux module Mo P13U, 30 nm pores size, Berghof, Germany) and exposed to an ultraviolet sterilization system (UV C, Purion® 500 Pro, Germany) within the building. All experiments were performed in triplicate.

The first repeated batch experiment was conducted in a multi-cultivator system (MC 1000-ODMC 1000-OD, Photon System Instrument, Czech Republic). Three different media compositions were used: A. Sterilized BIQ wastewater; B. Unsterilized BIQ wastewater; C. Control (Ferty Basis 1, Planta Düngemittel GmbH, Germany, Table 5). The control medium was supplemented with NH_4NO_3 as the TN source, referring to the TN concentration in the BIQ wastewater. After each repeated (refill) period, 10% of the algae culture volume was re-used and 90% of new fresh medium was added. In this experiment, parameters observed were optical density, cell dry weight, and photosynthetic activity. For the second refill, elements that would cause growth limitation (i.e. Mg, Fe and Mn) were supplemented to media A and B. Magnesium, manganese, and iron were supplemented referring to the Ferty Basis 1 medium.

A modified experiment was conducted to recheck the results of the first repeated batch experiment. Therefore, 3 different media compositions were applied: A.BIQ wastewater with only 3 elements limiting growth (Mg, Mn, Fe, referred to Ferty Basis 1); B. BIQ wastewater with all micro elements (referred to the Ferty Basis 1) and C. Control (Ferty Basis 1, Planta Düngemittel GmbH, Germany). The TN source in the control medium was obtained from NH_4NO_3 at the same concentration as the first repeated batch experiment. Like the parameters measured in the first repeated batch, growth was followed by OD and CDW while photosynthetic activity was measured on the same day as OD determination. This recheck experiment was carried out in the above-described multi-cultivator system (MC 1000-ODMC 1000-OD, Photon System Instrument, Czech Republic).

A second series of repeated batch experiment was performed using a Kniese system with a working volume of each tube of 350 ml (Figure 4). Three different media compositions were applied: A. BIQ wastewater; B. BIQ wastewater with Mn, Mg and Fe; C. Control (Ferty Basis 1, Planta Düngemittel GmbH, Germany). The TN source in the control medium was obtained from NH_4NO_3, referring to the concentration within the BIQ wastewater used in this experiment. For each refill period, all cultures were primarily centrifuged (Heraeus Mega Fuge 11R, Thermo Scientific, USA) for 10 minutes at 4000 rpm, the supernatant was disposed and cells were resolved in the new media composition. Parameters measured in this experiment were OD, CDW, photosynthetic activity, pH, Total Nitrogen (TN), Total Phosphorus (TP),

Dissolved Organic Carbon (DOC) and Chemical Oxygen Demand (COD) -except in media C, where COD was not observed. Elements supplied to media B were concurrent with the element composition in Ferty Basis 1 medium.

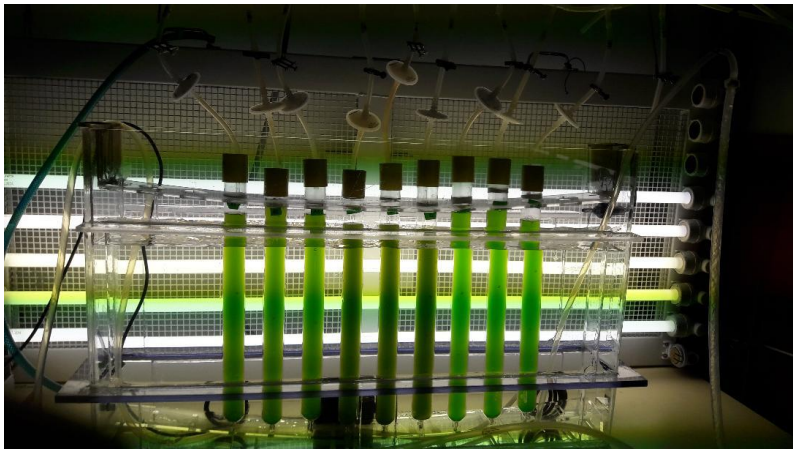


Figure 4. Kniese system for alga cultivation

Table 5. Composition of Ferty Basis 1 medium

Compound	%
Phosphorus pentoxide (P_2O_5)	14
Potassium oxide (K_2O)	38
Magnesium oxide (MgO)	5
Sulphur trioxide (SO_3)	35
Boron (B)	0.02
Copper as chelate of EDTA	0.003
Iron as chelate of DTPA (0.14%) and EDDHA (0.06%)	0.2
Manganese as chelate of EDTA	0.04
Molybdenum	0.006
Zink as chelate of EDTA	0.005

2.2.4. Trophic modes experiment

The experiment was carried out in the Kniese system and consisted of 4 different media, light and gas supply compositions (Figure 5). Before the experiment started, media B, C and D were acidified until pH 3 was achieved and purged with N_2 for 10 minutes. Afterwards, pH was adjusted to 7.5 while still purging with N_2. The pH adjustment was carried out with 10% and 25% of HCl and 1 M and 10 M of NaOH to keep the value close to 7.5. A detail design of the experiment is presented in Table 6. Approach D had a special treatment as tubes were covered with black paper to avoid light penetration. The BIQ wastewater used in this experiment was collected at a different period compared to that used in previous experiments. During the 10 days of the experiment, CDW, OD and photosynthetic activity of *A. obliquus* was determined every day. Adjustment of pH was carried out every day with NaOH and HCl to keep the value

close to 7.5. In addition, oxygen evolution and respiration rates were examined every day. Detailed measurements of oxygen evolution and respiration rates were explained in sub-chapter 2.3.7. Referring to KC, 3 elements that showed growth limitation were added in media A, B and D. In medium D, the culture was purged with synthetic air, a gas mixture of nitrogen and oxygen, which is free of hydrocarbon.

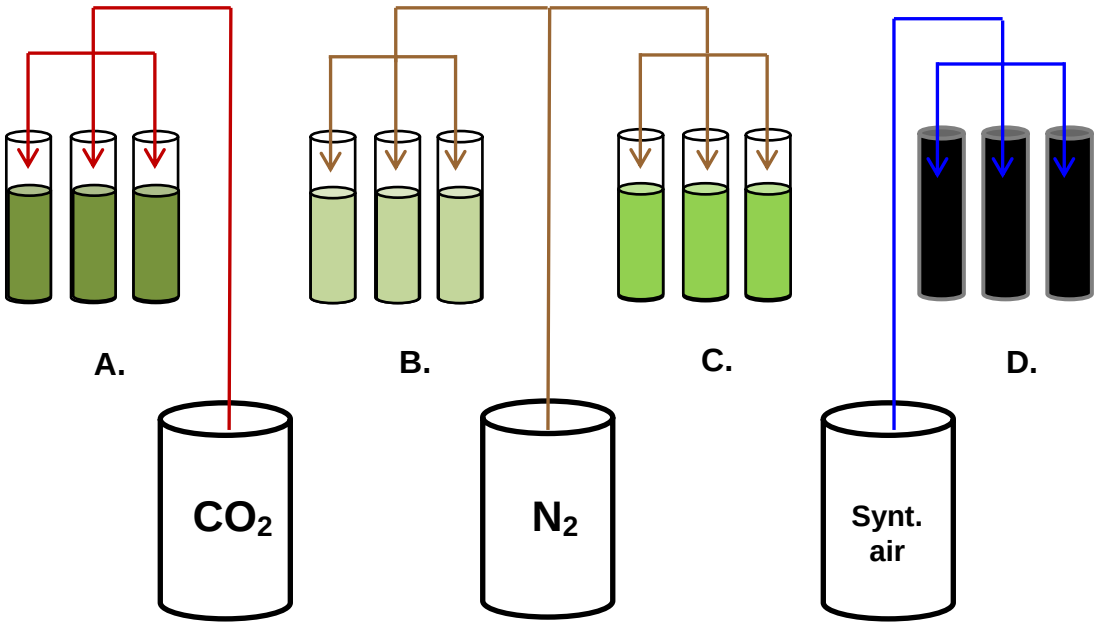


Figure 5. Schematic diagram of experimental design of the trophic mode experiment

Table 6. Detailed information of experimental design of a trophic mode experiment

		Trophic Mode	Energy source	Carbon source
A.	WW+CO_2+light	Mixotrophic	Light, Organic C (wastewater)	Inorganic (CO2) and Organic (wastewater)
B.	WW+N_2+light	Photoheterotrophic-1	Light, Organic C (wastewater)	Organic (wastewater)
C.	Modif. KC+light+N_2	Photoheterotrophic-2	Light, Organic C (acetic acid)	Organic (acetic acid)
D.	WW+synthetic air	Heterotrophic	Organic C (wastewater)	Organic (wastewater)

2.2.5. Outdoor Experiment

Two outdoor experiments were conducted during different time periods. The first outdoor experiment was performed in August 2016 (summer), when the average maximal irradiance every day was above 900 µmol photon m^2 s^{-1}. The second outdoor experiment was performed in October 2016 (autumn), when the average maximal irradiance over 8 from 14 days of the experiment was lower than 400 µmol photon m^2 s^{-1}. Both experiments were performed in a flat panel photobioreactor façade system, installed on the BIQ apartment building (Figure 6). The

photobioreactor façade system consisted of 129 vertical flat panels, facing the southwest and the southeast sides of the building and covering a total area of 200 m^2. Four independent lines of photobioreactors were located on the facades, named L1, L2, L3 and L4 (from the ground to the top floor). The maximal volume of each line was 1000 L.

As described in Figure 7, the flow of BIQ wastewater started from its collection through a piping system to an anaerobic membrane bioreactor (AnMBR) tank with a volume of 800 L. Ten days of hydraulic retention time (HRT) was used for anaerobic digestion in the tank. Further on, the wastewater flowed to an ultrafiltration membrane and was exposed to an ultraviolet sterilization system, as also described in sub-chapter 2.2.3 and Figure 7. Subsequently, the BIQ wastewater was collected in a flotation tank to be mixed with a culture of *A. obliquus.* Before being distributed to the photobioreactors, the culture flowed through a heat exchanger, a sensors section unit and a carbon dioxide (CO_2) saturation device (Figure 8). Carbon dioxide (CO_2) was supplied from flue gas, which was generated from a centralized gas burner system. Afterwards, gas was streamed through a membrane system before it was collected in a saturation device. Each line of photobioreactor façade had its own pump and circulation system as well as an interconnection system (Figure 8). The ground line of photobioreactors (L1) was used as a stock culture for the experiment, with an artificial fertilizer as a culture medium (Ferty Basis 1, Planta Düngemittel GmbH, Germany). The experimental designs for both experiments are described in Table 7. In the experiments, OD, CDW, photosynthetic activity, TN and TP in the medium were measured. In addition, DOC and COD were also observed.

Table 7. Experimental design of 2 batch outdoor experiments at the BIQ Alga House

	L2	**L3**	**L4**
High light outdoor experiment	Control (Ferty Basis 1)	BIQ wastewater + Mn, Mg and Fe	BIQ wastewater
Low light outdoor experiment	BIQ wastewater	BIQ wastewater + Mn, Mg and Fe	Control (Ferty Basis 1)

In the high-light outdoor experiment, each line was filled up to 700 L. Total nitrogen and phosphorous concentrations of the BIQ wastewater and pre-culture were 61.59 and 3.38 mg L^{-1} and 10 and 15.8 mg L^{-1}, respectively. After calculation, 508.84 L of BIQ wastewater was mixed with 191.16 L of pre-culture to achieve an N:P ratio of 7 at the beginning of experiment. Elements added in L3 were adjusted according to the limiting growth concentrations.

Each line in low-light experiment was filled up to 500 L. The nutrient concentrations of the BIQ wastewater and pre-culture in this experiment were 94 and 7.05 mg L^{-1} and 9.13 and 50.8 mg L^{-1}, respectively for TN and TP. By calculation, 444 L of BIQ wastewater was mixed

with 56 L of pre-culture. Hence, an N:P ratio of 7 was achieved. Elements added in L3 were adjusted according to the limiting growth concentrations.

Figure 6. Bio Intelligence Quotient (BIQ) House apartment building in Wilhemsburg, Hamburg, Germany

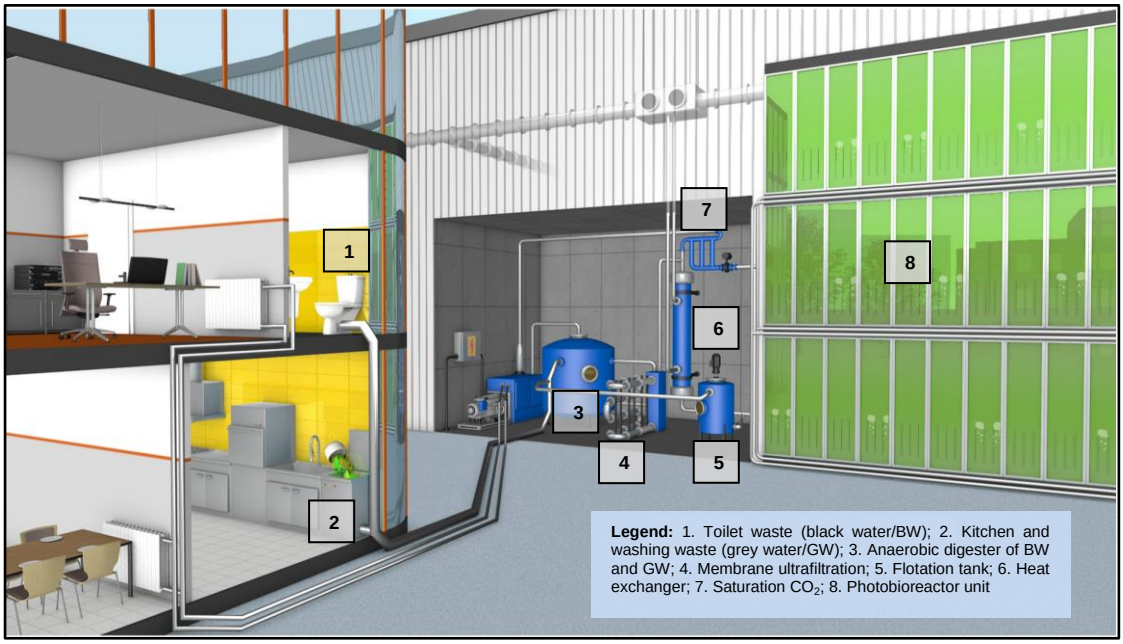


Figure 7. The wastewater utilization for energy resource and nutrients recycling concept (modified from SSC, Strategic Science Consult GmbH)

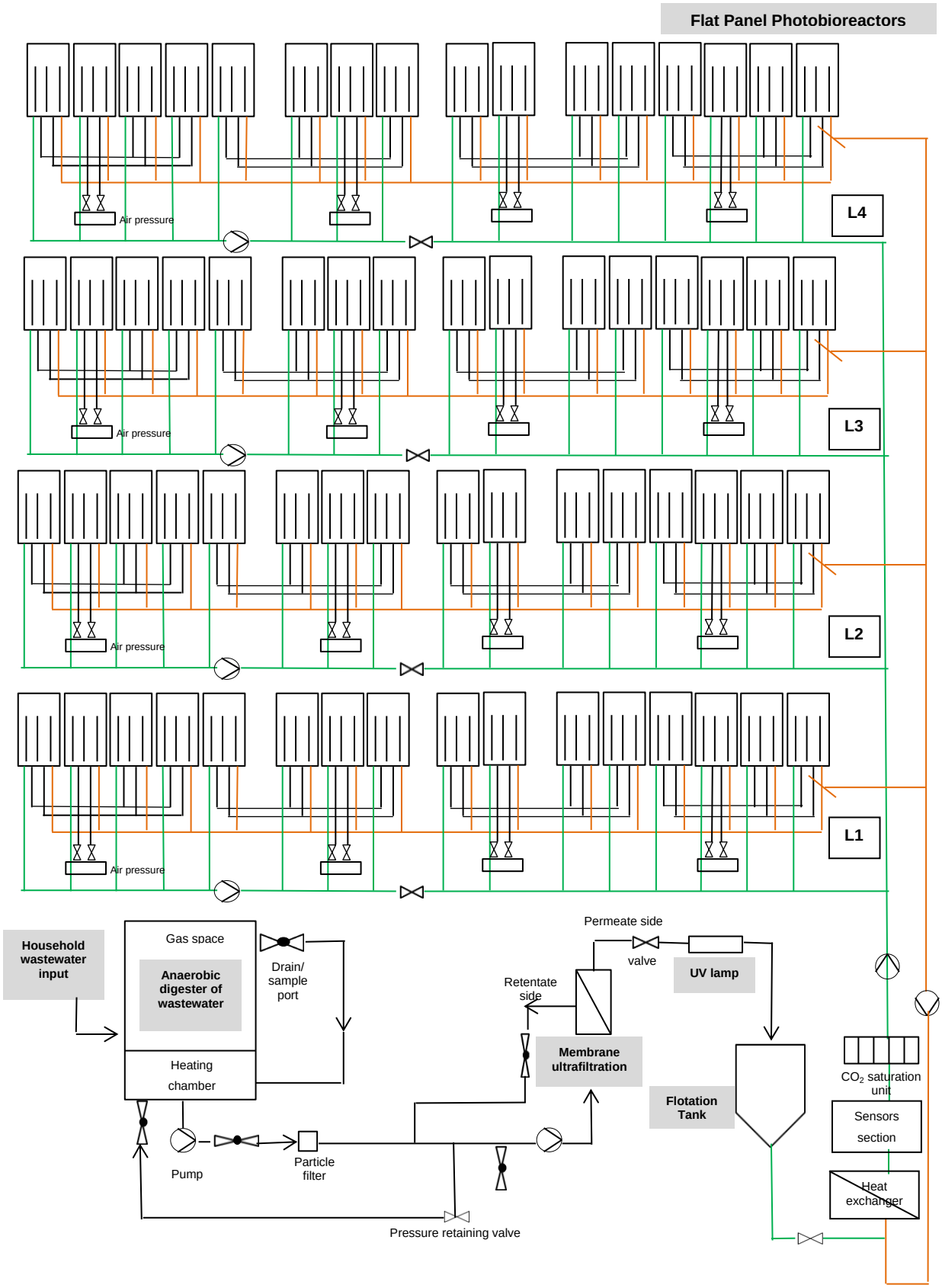


Figure 8. Schematic flow diagram of the BIQ wastewater and the alga culture during the outdoor experiments at the BIQ building (modified from technical drawings prepared by SSC Strategic Science Consult GmbH and TU Berlin)

2.3. Methods

2.3.1. Algal growth

Biomass production was calculated with cell dry weight and optical density. Cell dry weight (CDW) was measured with 1.2 µm pore size membrane filter (GF/C, Whatman, Germany). Prior to measurement, filters were dried in an oven at 80^0C overnight. A certain amount of sample volume was filtered, after which the filters were dried in the drying oven overnight. Biomass concentration was calculated based on the volume or illuminated area. The biomass concentration was calculated as follows:

$$CDW\ (g\ L^{-1}) = \frac{W_1 - W_0}{V} \times 1000 \quad (1)$$

where : W_1 = dry weight of filter and biomass (g)

W_0 = dry weight of filter (g)

V = volume of the filtered sample (ml)

Areal biomass concentration was calculated as follows (Wen et al. 2016):

$$Areal\ biomass\ concentration\ (C_{biomass}, g\ m^{-2}) = \frac{CDW \times V}{S} \quad (2)$$

where : V = volume of the culture (L)

S = illuminated area (m^{-2})

Productivity of *A. obliquus* was calculated based on the volume of the culture or illuminated area of the photobioreactor. Productivity was calculated as follows:

$$Productivity\ (P, g\ L^{-1}\ d^{-1}\ or\ g\ m^{-2}\ d^{-1}) = \frac{CDW_1\ (C_{biomass\_1)} - CDW_0\ (C_{biomass\_0)}}{t_1 - t_0} \quad (3)$$

where : t_1 = culture time (day-1)

t_0 = culture time (day-0)

According to Andersen (2005), specific growth rate was calculated based on cell dry weight as follows:

$$\mu\ (d^{-1}) = \frac{lnCDW_{t1} - lnCDW_{t0}}{t_1 - t_0} \quad (4)$$

where CDW_{t1} and CDW_{t0} were the CDW at the end and beginning of an exponential phase and t_1-t_0 was the time interval during the exponential phase.

Optical density was measured at 680 and 750 nm with a UV-VIS spectrophotometer (UV-2401 PC, Shimadzu, Japan). About 1 ml of volume was taken every day from each tube of the experiment to observe the optical density of the culture.

2.3.2. Photosynthetic activity

Photosynthetic activity of the cell was measured by the maximum quantum yield of photosystem II. The measurement was based on the saturation pulse method, applying a strong pulse of light (8000 μmol photon m^2 s^{-1}) to an algal culture (1 ml volume) after a pre-dark adaptation period of 10 minutes. This causes the saturation of the photochemical status of photosystem II (PS II). As photochemical quenching is still zero, energy dissipation by fluorescence and heat production become maximal. The ratio of the difference of maximal (Fm) and minimal (Fo) divided by maximal (Fm) fluorescence (Fv/Fm) was used to measure photosynthetic activity in vivo (Hanelt 1992).

The photosynthetic activities of cells were measured every 24 hours with a Pulse Amplitude Modulation (PAM) Fluorometer (Imaging PAM, Heinz Walz GmbH, Germany).

2.3.3. Nutrient Analysis

Major nutrients analyzed in this study were total nitrogen (TN, inorganic and organic), ammonium nitrogen (NH_4-N), nitrate nitrogen (NO_3-N), total phosphorus (TP), phosphorus in a phosphate form (PO_4-P), total carbon (TC, inorganic and organic), total organic carbon (TOC) and dissolved organic carbon (DOC). Elements measured were: potassium (K), sodium (Na), magnesium (Mg), calcium (Ca), iron (Fe), manganese (Mn) and boron (B).

2.3.3.1. Determination of nitrogen content

Total nitrogen was analyzed with a TOC analyzer (TOC-VCPN, Shimadzu, Japan), equipped with a total nitrogen measuring unit (TNM-1, Shimadzu, Japan). Total nitrogen samples were analyzed at the beginning and end of an exponential phase during the experiments. Nitrogen removal was measured from the pre-filtered culture medium. The culture medium was filtered with a 0.45 μm membrane filter (Whatman, 7404-004, Germany). TN removal was further calculated as follows:

$$TN_{removal}(mg\ L^{-1}) = TN_0(mg\ L^{-1}) - TN_1(mg\ L^{-1}) \quad (5)$$

where TN_1 is total nitrogen in the medium at the end of the exponential phase and TN_0 is the total nitrogen in the medium in the beginning of exponential phase. The percentage of TN removal was further calculated as follows:

$$TN_{removal}(\%) = \frac{TN_{removal}\,(mgL^{-1})}{TN_0(mgL^{-1})} \times 100\% \quad (6)$$

Total nitrogen content in cells was calculated from the total TN in the culture subtracted by the TN in the filtered medium. Total nitrogen uptake by cells was further calculated as follows:

$$TN_{cells\_uptake}(mg\ g^{-1}CDW) = \frac{TN_{cells\_t1} - TN_{cells\_t0}}{increased\ CDW} \quad (7)$$

where TN_{cells_t0} and TN_{cells_t1} are the TN in cells in the beginning and at the end of exponential phase, respectively.

Ammonium nitrogen (NH_4-N) was measured photometrically with a cuvette test (LCK 305 or LCK 303, Hach Lange, Germany) while nitrate nitrogen (NO_3-N) concentration was measured with a different cuvette-test (LCK 339 or LCK 340, Hach Lange, Germany). Analysis was performed by using a photometer (DR-3900, Hach Lange, Germany).

2.3.3.2. Determination of phosphorus content

Total phosphorus (TP, inorganic and organic) and phosphorus in phosphate form (PO_4-P) concentrations were photometrically analyzed with a phosphate (ortho/total) cuvette test (LCK 349 and LCK 350, Hach Lange, Germany). To measure TP and PO_4-P in the medium, the sample was pre-filtered using a 0.45 μm membrane filter (Whatman, 7404-004, Germany). To also calculate TP in cells, unfiltered samples were also measured. Total phosphorus content in cells was calculated from the TP in the culture subtracted by the TP in the medium.

Total phosphorous removal from the medium was calculated as follows:

$$TP_{removal}(mg\ L^{-1}) = TP_0(mg\ L^{-1}) - TP_1(mg\ L^{-1}) \quad (8)$$

where TP_1 is the total phosphorus in the medium at the end of exponential phase and TP_0 is the total phosphorus in the medium at the beginning of exponential phase.

The percentage of TP removal from the medium was calculated as follows:

$$TP_{removal}(\%) = \frac{TP_{removal}\,(mgL^{-1})}{TP_0(mgL^{-1})} \times 100\% \quad (9)$$

Total phosphorus content in cells was calculated from the total TP in the culture subtracted by the TP in the filtered medium. Total phosphorus uptake by cells was further calculated as follows:

$$TP_{cells\_uptake}\ (mg\ g^{-1}CDW) = \frac{TP_{cells\_t1} - TP_{cells\_t0}}{increased\ CDW} \quad (10)$$

where TP_{cells_t0} and TP_{cells_t1} are respectively the TP in cells at the beginning and end of the exponential phase.

2.3.3.3. Determination of carbon content

In this study, carbon concentrations were measured as total carbon (TC), total organic carbon (TOC) and dissolved organic carbon (DOC). Measurements were performed with an oxidative combustion analysis method in a TOC analyzer (TOC-V_{CPN}, Shimadzu, Japan). To measure TC and TOC, each sample was prepared twice, first to measure TC and second to measure TOC. TC was measured without acidification. TOC was measured by acidifying the sample to pH 2-3 to eliminate inorganic carbon compounds from the sample. Therefore, only TOC remained in the sample. To measure DOC, the sample was pre-filtered with a 0.45 μm membrane filter (Whatman, 7404-004, Germany).

2.3.3.4. Determination of elements

Analysis was performed by using inductively coupled plasma mass spectrometry (ICP-MS) with a collision/reaction cell (Agilent 7700 cx, Agilent Technologies, Tokyo, Japan). Elements were only analyzed in the supernatant of the cultures. The supernatant was obtained by filtering the culture medium with a 0.45 μm membrane filter (Whatman, 7404-004, Germany). Before analysis, samples were diluted with MilliQ Water and acidified with sub-boiled nitric acid. Elemental analysis in this study was carried out by Dr. Daniel Proefrock from the Zentrum für Material- und Küstenforschung, Helmholtz Zentrum Geesthacht (HZG), Institute for Coastal Research/Operational Systems, Department for Marine Bioanalytical Chemistry, Geesthacht, Germany.

2.3.4. Pigment determination

Pigment analysis was initiated with a pigment extraction method from the alga biomass (Patzelt 2016, personal communication). One ml of alga culture (about 0.5 g L^{-1}) was centrifuged in a 2 ml microtube (Ref.72.608, Sarstedt, Germany) at 9500 g (10000 rpm) for 4 minutes (Biofuge pico, Heraeus Instrument, Germany). Subsequently, the supernatant was decanted. Glass beads of 1.7-2 mm and 0.25-0.3 mm (Kat.Nr. 54160, B Braun Melsungen AG,

Germany) of 0.097 and 0.267 gr, respectively, were added to the tube with an ethanol pre-washed spoon. Subsequently, cells were disrupted in a homogenizer (Bead Blaster™ 24, Benchmark, USA) at maximum speed for 5 cycles, 55 seconds each. To extract the pigments, 1 ml of ethanol (Chromasolv® for HPLC, absolute, ≥99.8%, Sigma Aldrich, Germany) was added to samples and further homogenization was carried out in the homogenizer at the same speed, duration and cycle periods with the previous homogenization step. To separate the ethanol extract from the cell debris, the aliquot was further centrifuged at 9500 g (10000 rpm) for 4 minutes (Biofuge pico, Heraeus Instrument, Germany). Extracted pigments were taken up with 1 ml syringes, filtered with 0.45 membrane filters (Multoclear-13 PTFE CS, Chromatographie Service GmbH, Germany) and placed in micro-insert tubes (G30ls, Art-Nr. 300407, Chromatographie Service GmbH, Germany). Subsequently, the tubes were placed into 1.5 ml HPLC vials (flat screw neck, 11.6 x 32 mm/clear, 8-425, Macherey-Nagel GmbH & Co. KG, Germany) and closed with screw caps (N8 black/center hole white, PTFE red 1.3 mm, Macherey-Nagel GmbH & Co. KG, Germany) for further analysis. During transportation, samples were always covered in a dark container with ice to prevent pigment destruction by light and to reduce heat after homogenization.

Extracted pigments were subsequently analyzed using a high performance liquid chromatography (HPLC) system (Elite LaChrom, VWR Hitachi, Japan). The HPLC consisted of a pump, an autosampler, a column oven and a diode array detector (DAD). The column used was a reversed phase RP-18 (LiChrosphere®100, 5 μm, LiChroCART® 250-4). The flow rate was 0.5 ml $min.^{-1}$ and the injection volume was 10 μl. Analysis was performed by using a gradient of two solvent compositions, named as solvent B (methanol: tetrahydrofuran (THF): acetonitrile = 15%: 10%: 75%) and solvent A (80% of solvent B and 20% of distilled water), following the gradient in Table 8. Pigments were detected at 436 nm, according to Andersen (2005). Eluents were sampled by an autosampler and later analyzed spectrophotometrically. Pigment identification was performed using retention time, peak and shape of the spectra from the DAD, in comparison with commercial standards (DHI Lab.Products, Denmark).

Table 8. Gradient used in the HPLC analysis

Time (minutes)	A (80% from B)	B (100%)
0	100	0
1	100	0
15	15	85
35	0	100
39	100	0

2.3.5. Transmission electron microscopy (TEM) determination

The inner structure of the *A. obliquus* cells was observed by transmission electron microscope (Leo 906 E TEM, Oberkochen, Germany) equipped with a multiscan CCD Camera

(model 794, Gatan, Munich, Germany). Further observation was performed using software (digital micrograph software version 2.0.2., Gatan, Munich, Germany) to acquire, visualize, analyze and process image data. This analysis method refers to Quader (1984). Fixation was carried out with 2% glutaraldehyde in cacodylate buffer (75mM, pH 7) for 3 hours. One percent of osmium tetroxide was used overnight at 4^0C, for a post fixation process. A series of graded acetone concentrations (30-100%) were used for dehydration process. Subsequently, samples were embedded in plastic, according to Spurr (1969). To acquire ultrathin sections, an ultramicrotome (Ultracut E, Leica-Reichert_Jung, Nußloch, Germany) was used and subsequent staining was performed with uranyl acetate and lead citrate (Reynolds 1963). Sample preparation was performed together with the technical assistant Elke Woelken.

2.3.6. Determination of chemical oxygen demand

Chemical oxygen demand (COD) analysis was photometrically performed with a cuvette test (LCK 314 and LCK 414, Hach Lange, Germany). COD consumption was measured from the filtrate of the culture medium. Hence, samples were pre-filtered with 0.45 μm membrane filter (Whatman, 7404-004, Germany) prior to measurements.

2.3.7. Oxygen evolution and respiration rate measurement

Prior to the measurement, a built-in measurement chamber (Figure 9) was prepared from a quartz glass cuvette (Type No. 101-QS, Quartz glass Suprasil, Hellma, Germany). In the inner wall of the cuvette, an oxygen sensor spot (SP-PSt3-NAU-D5-YOP, PreSens GmbH, Germany) was attached with silicone glue (RS 892-542, RS Components Ltd., UK). In addition, a magnetic stirrer of 3 mm in diameter and 8 mm length (VWR, Germany) and a built-in stopper (ϕd1:14 mm; ϕd2:18 mm; h:20 mm, Deutsch & Neumann, Germany) were prepared.

Figure 9. A built-in chamber for oxygen evolution and respiration rate measurements

Oxygen evolution and respiration rates were measured with a fiber optic oxygen meter (Fibox 3, PreSens GmbH, Germany) equipped with a software (Oxy View 3.51, PreSens, GmbH, Germany) to record the data. Before measurement started, calibration was performed.

Initially, 4 ml of culture with a known cell dry weight was filled in the measurement chamber. The culture was subsequently purged with argon (Ar) gas for 5 minutes, immediately closed with the rubber stopper, and kept under dark condition for 5 minutes until a constant oxygen concentration was reached as a baseline. Oxygen evolution was measured under illumination of 420 μmol m^{-2} s^{-1} for a period of 5 minutes, followed by another 5 minutes in the dark, respectively equal to oxygen evolution and respiration rates. The intensity of light used (420 μmol m^{-2} s^{-1}) was the same as the light intensity used in the previous experiment with BIQ wastewater. At that light intensity, optimal growth of *A. obliquus* was achieved. The light source used was obtained from a slide projector (Leitz, Wetzlar, Prado Universal) equipped with a halogen bulb (24 V / 250 W).

2.3.8. Statistical analysis

All statistical analysis was carried out using SPSS Statistic 23 (IBM, USA). Results were obtained from the mean ± standard deviation (SD) of the triplicate. Significant differences were tested using analysis of variance (ANOVA) at a significance level of $\alpha=0.05$. A Tukey HSD post hoc test was used for a multiple group comparison.

3. Results

3.1 Laboratory scale

3.1.1 Cultivation in black water

3.1.1.1 Characteristics of the black water

Compared to its original condition, only about 30% and 24% of total carbon (TC) and total organic carbon (TOC), respectively, remained after anaerobic digestion, filtration with 0.1 µm pore size and sterilization with ultraviolet radiation. However, about 76% and 97% of total nitrogen and N-ammonium remained and about 35% and 90% of total phosphorous and P-phosphate was observed (Table 9). The concentration of total nitrogen in the black water used in this experiment (1300 mg L^{-1}) was similar to other anaerobic digested black water from reference experiments (between 1100-1900 mg L^{-1}), but the total phosphorous concentration (61.4 mg L^{-1}) was below the range of the total phosphorous concentration determined in anaerobic digested black water described in references (between 105.2-280 mg L^{-1}) (Table 9).

Table 9. Comparison of Carbon, nitrogen and phosphorus concentration in BW used for the experiment and concentration in BW from other references

Parameter	Raw BW (mg L^{-1})	BW after anaerobic digestion, filtered and UV exposed (mg L^{-1})	BW after anaerobic digestion (mg L^{-1}) (Fernandes et al. 2015)	BW after anaerobic digestion (mg L^{-1}) (Zeeman et al. 2008)
TC	6750	2010	-	-
TOC	5780	1380	-	-
TN	1720	1300	1210	1100-1900
NH_4-N	1140	1110	1070	710-1400
NO_3-N	6.18	<0.5	-	-
TP	176	61.4	105.2	110-280
PO_4-P	64.3	57.7	73	-

3.1.1.2 Optimal growth concentration

To determine optimal nutrient concentration for the growth of *A. obliquus* using BW, 3 different BW concentrations were used (Figure 10). In the first 8 days of the experiment without additional elements *A. obliquus* only grew in the control medium, which contained artificial fertilizer (Flory Basisdünger 1). The exponential phase of *A. obliquus* in the control medium began at day-2 and continued until the end of experiment. Addition of all micro elements from Kessler, E. und Czygan (1970) at day-8 initiated the growth of *A. obliquus* in all concentrations of BW media.

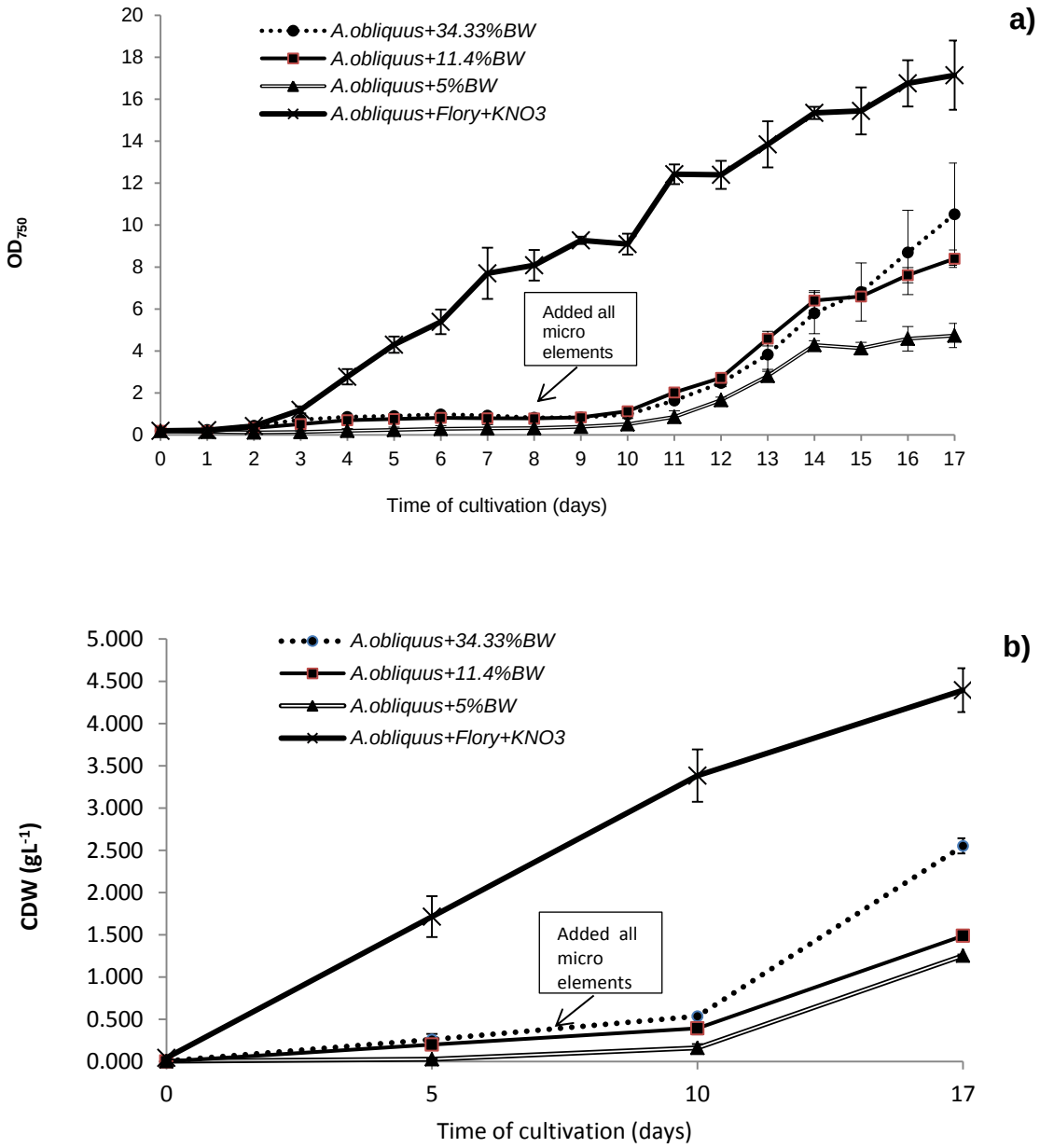


Figure 10. Growth of *A. obliquus* in different media compositions of black water and in a control medium (artificial fertilizer) monitored by: a) Cell dry weight and b) Optical density. Mean values were calculated from triplicate ± standard deviation.

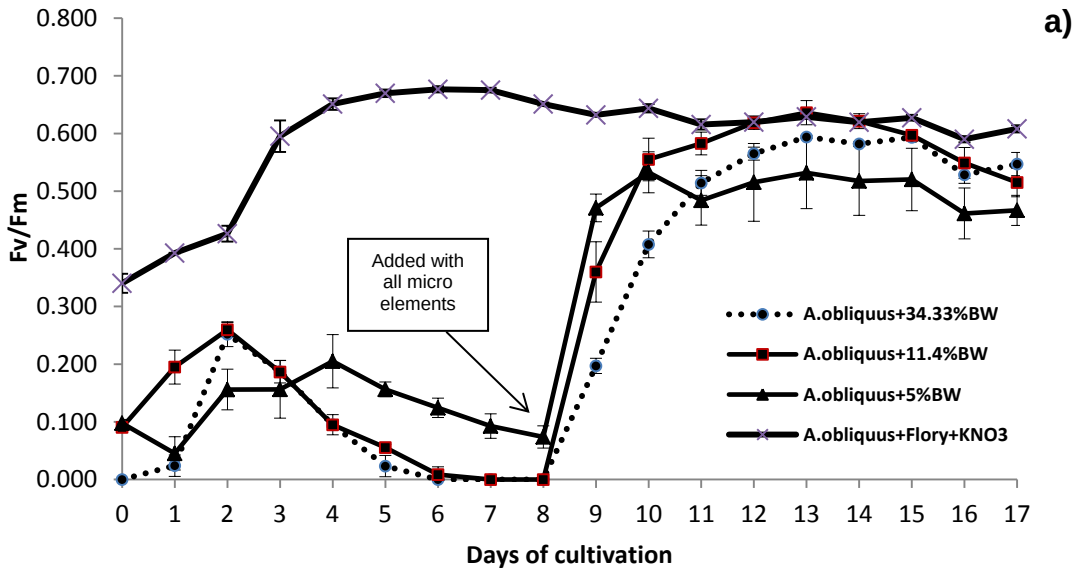
Although productivity of *A. obliquus* after addition of micro elements in 34.33% of BW of 0.288 g L^{-1} d^{-1} was only 86.22% than that of control, no statistically significant difference was observed (ANOVA F=41, d.f. 3,8, $p \geq 0.05$) (Table 10). However, the productivity achieved in 34.33% BW was 1.83 and 1.85 times higher than those in 11.4% and 5% BW, respectively.

Table 10. Productivity of *A. obliquus* in different media compositions of BW and in a control medium

	Culture conditions	Mean Cell Dry Weight (CDW) at the early exponential phase (gL^{-1})	Mean (CDW) at the end of exponential phase (gL^{-1})	Mean Productivity ($g\ L^{-1}\ d^{-1}$)
I.	34.33% BW	0.536 ± 0.013	2.552 ± 0.090	0.288 ± 0.013[a]
II.	11.4% BW	0.393 ± 0.072	1.489 ± 0.054	0.157 ± 0.003[b]
III.	5% BW	0.161 ± 0.045	1.253 ± 0.014	0.156 ± 0.008[b]
IV.	Flory+3.22 g KNO3	0.043 ± 0.010	1.715 ± 0.242	0.334 ± 0.047[a]

Mean values were calculated from triplicate ± standard deviation. Upper case letter of each mean productivity value shows statistically significant differences at $p \leq 0.05$ (ANOVA).

Photosynthetic efficiency (Fv/Fm) of *A. obliquus* in this experiment was partly in accordance with growth results. At the beginning of the experiment, Fv/Fm values in all media with BW were very low (0-0.135) then started to increase at day-1 and continue until day-2 in 34.33% BW and 11.4% BW and until day-4 in 5% BW. At the beginning of experiment, the Fv/Fm value of *A. obliquus* in control medium was higher than in BW and started to increase until day-6 (Figure 11-a). From day-7 until the end of experiment, the Fv/Fm value in control medium was relatively constant (0.59-0.677). The Fv/Fm values of *A. obliquus* from day-4 until day-8 in 5% BW showed less stress (0.074-0.205) than in 11.4% BW and 34.33% BW (0-0.0959). However, after micro elements were added at day-8, the photosynthetic efficiency of *A. obliquus* in three different concentrations of BW began increasing strongly with the highest mean range values of 0.36-0.636 observed in 11.4% BW (Figure 11a).



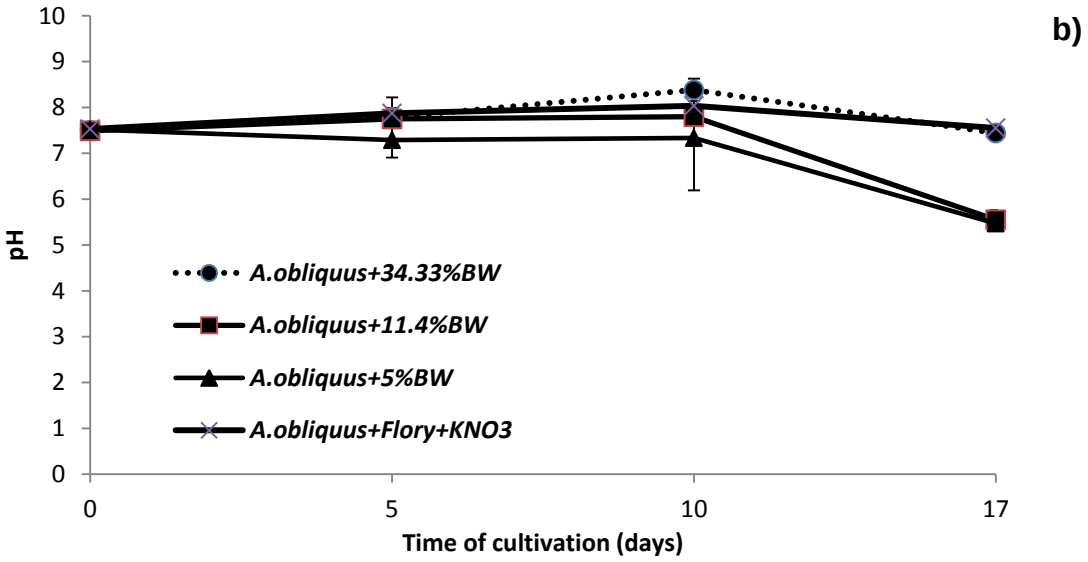


Figure 11. a) Photosynthetic efficiency of *A. obliquus* (Fv/Fm) and b) pH values during growth in different media compositions of black water and in a control medium. Mean values were calculated from triplicate ± standard deviation.

Relatively constant pH values were observed in the culture with 34.33% BW (7.5-8.38) and in the control (7.53-8.04). The highest pH value in the culture with 34.33% BW and the control was achieved at day-10 after elements were added at day-8. In contrast, pH values in the cultures with 11.4% BW and 5% BW decreased 2 days after the addition of elements (Figure 11b).

3.1.1.3 Element limitation experiment

3.1.1.3.1 Growth and photosynthesis

To examine which elements were missing to support growth of *A. obliquus* in BW, elemental composition in cultivation medium from Kessler, E. und Czygan (1970) was used for comparison. Elemental analysis of BW showed that there were mainly 3 elements: magnesium (Mg), manganese (Mn) and iron (Fe), with concentrations lower (about 27.18%, 14.39% and 10%, respectively) than those in the Kessler, E. and Czygan (KC) medium (Table 11).

Table 11. Comparison of some elements in BW and elements used in control medium (Kessler and Czygan, 1970)

Elements	BW used in this study after anaerobic digestion (mg L^{-1})	Raw BW-1 (mg L^{-1}) (Palmquist and Hanæus 2005)	Raw BW-2 (mg L^{-1}) (Brandes 1978)	KC (mg L^{-1})	In culture medium of 34.33% BW (mg L^{-1})
K	219.2	75	41	313.233	75.260
Na		97.7		277.257	
Mg	6.7	17	4	24.653	2.300
Ca	19.28	68.6	29	3.817	6.618
Cl			98	288.570	
Fe	0.12	1.28	1.2	1.205	0.041
Mn	0.02	0.130		0.139	0.008
B	1.8			0.087	0.618
Zn	0.04	0.525		0.045	0.013
Mo				0.00155	

The availability of potassium (K) and zinc (Zn) in 34.44% BW was only about 24% and 29% of that from KC medium. However, both concentrations were still higher than Mg, Mn and Fe, with concentrations of less than 10% of those in the KC medium (Table 11). Hence, K and Zn were not added to the BW culture medium to minimize the utilization of additional element sources.

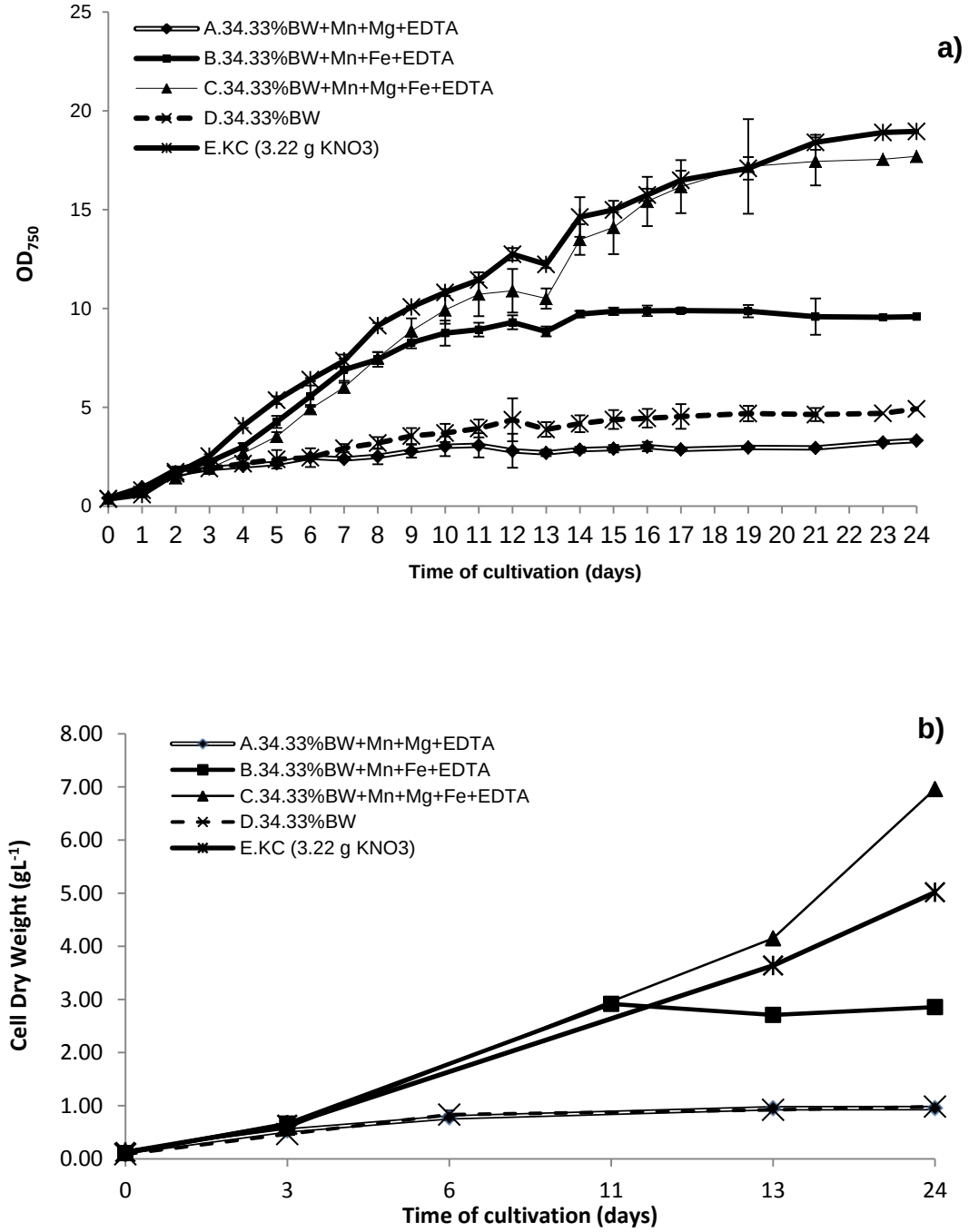


Figure 12. Growth of *A. obliquus* in 34.33% BW with different element composition and in a control medium (KC), based on: a) Optical density and b) Cell dry weight. Mean values were calculated from triplicate ± standard deviation. KC medium was modified to equalize its nitrogen content to BW.

The growth of *A. obliquus* in 4 different media compositions of BW and 1 control medium (KC plus 3.22 g KNO_3) are shown in Figure 12. At the end of the experiment, optical density in medium C (34.33% BW+Mn+Mg+Fe) was 93.4% of the control medium but 1.85 times higher than medium B (34.33% BW+Mn+Fe) and 3.6-5.33 times higher than media D and A (Figure 12.). However, productivity s of *A.obliquus* in medium C was significantly 1.2 times higher than in the control (medium E) and there was no significant difference of productivities between medium A and medium D (ANOVA F=992, d.f. 4,10, p≥ 0.05) (Table 12).

Table 12. End biomass production and productivity of *A. obliquus* in different element compositions

	End Cell Dry Weight (g L^{-1})	Productivity (g L^{-1} d^{-1})
A.34.33%BW+Mn+Mg+EDTA	0.955 ±0.022	0.096 ±0.001[a]
B.34.33%BW+Mn+Fe+EDTA	2.855 ±0.056	0.313 ±0.012[b]
C.34.33%BW+Mn+Mg+Fe+EDTA	6.959 ±0.287	0.357 ±0.009[c]
D.34.33%BW	0.983 ±0.008	0.082 ±0.004[a]
E.KC (3.22 g KNO3)	5.170 ± 0.204	0.302 ±0.004[d]

Mean values are averaged from triplicate ± standard deviation. Upper case letter of each mean productivity value shows statistically significant differences at p ≤ 0.05 (ANOVA).

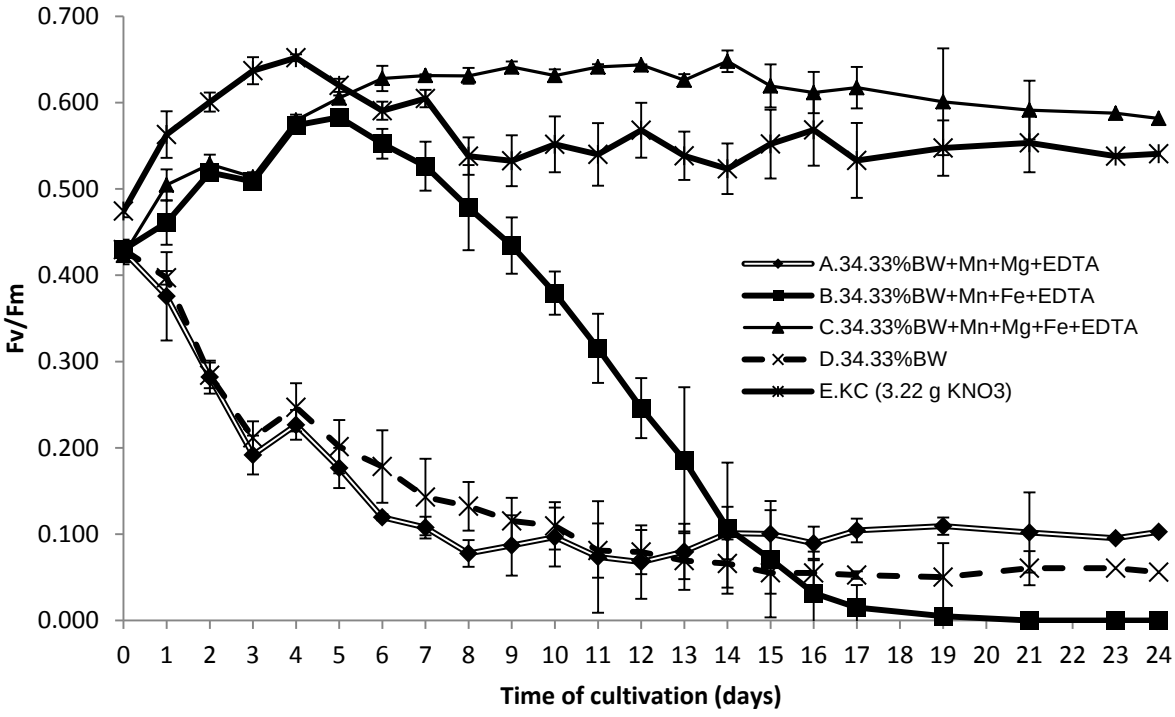
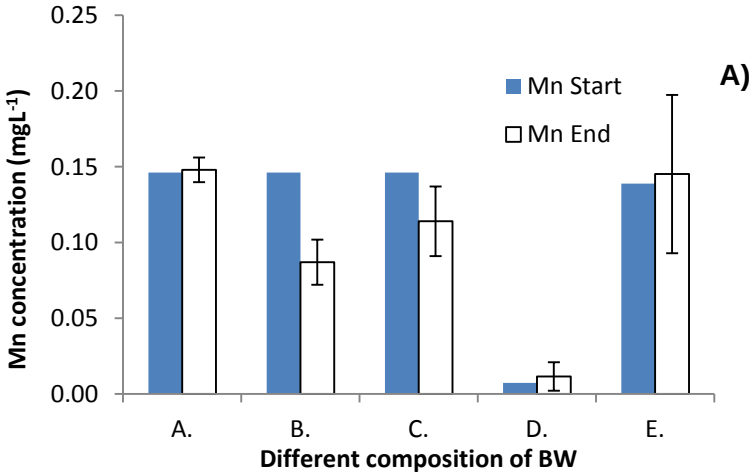


Figure 13. Photosynthetic efficiency of *A. obliquus* during the growth in four different element concentrations of BW and in a control medium. Mean values were calculated from triplicate ± standard deviation.

At the beginning of experiment, the Fv/Fm values of *A. obliquus* in all media compositions were relatively low (0.423-0.474), but it began to increase from day-1 until day-5 (in medium B), until day-9 (in medium C) and until day-4 (in medium E) (Figure 13). Until the end of the experiment, relatively stable Fv/Fm values (0.582-0.648) were observed in medium C. A slight decrease of Fv/Fm values in medium E from day-4 until day-8 (from 0.652 to 0.538) was observed, but the following Fv/Fm values were also relatively stable (0.523 - 0.568). Iron limitation induced the reduction of photosynthetic efficiency of *A. obliquus* observed in media A and D (Figure 13). The Fv/Fm values of *A. obliquus* in both media decreased from 0.428 to 0.068 (in medium A) and from 0.430 to 0.050 (in medium D). Although no magnesium limitation was primarily observed until day-5, subsequent reduction of Fv/Fm values from day-5 until the end of experiment (from 0.583 to 0) indicated a strong reduction of photosynthetic efficiency in medium B that may be initiated by magnesium limitation.

3.1.1.3.2 Limitation of micro elements

In the first analysis, growth limitation of *A. obliquus* by elements was calculated from the demand of different concentrations of the 3 elements of interest (Mn, Mg and Fe) in the medium. However, some results presented curiously higher concentrations of elements in the medium at the end, compared to the beginning of experiment (Figure 14). In medium C, in which *A. obliquus* grew optimally in BW (Figure 12), the iron concentration at the end of the experiment was 2.94 times higher than at the beginning (Figure 14b). Nevertheless, it can be deduced from Figure 14 that cells took up 0.032 mg L^{-1} Mn and 4.211 mg L^{-1} Mg from the medium in experiment C, when *A. obliquus* grew optimally in BW.



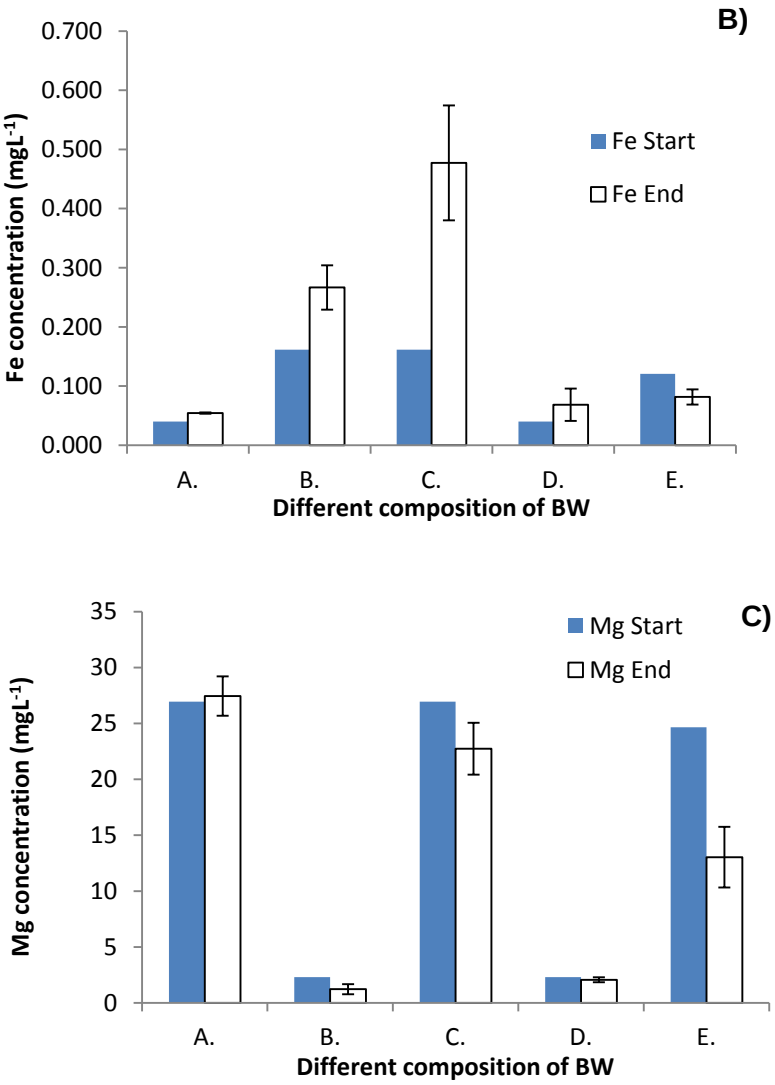


Figure 14. Concentration of: A). Mn; B). Fe and C). Mg in different compositions of BW media. Nomenclature according to Table 12. Mean values at the end of the experiment were calculated from triplicate ± standard deviation.

The second analysis was based on references by Elsayed et al. (2016) and Hecky and Kilham (1988). The results showed a calculated uptake of Mg, Fe and Mn of 27, 4.3 and 0.4 mg g^{-1}CDW, respectively, should occur during cell growth in medium C (Table 13). However, at the beginning of the experiment the optimal medium compositions of BW (medium C) consisted of Mg, Fe and Mn concentrations of only 26.953, 0.162 and 0.146 mg L^{-1}, respectively. This discrepancy will be discussed later.

Table 13. Magnesium, iron and manganese uptake concentration by cells cultivated in BW media calculated by the measured increase of CDW (modified from Elsayed et al. 2016**;** Hecky and Kilham 1988**).**

	Increase CDW ($g\ L^{-1}$)	Mg uptake ($mg.\ g^{-1}$ of total increase CDW)	Fe uptake ($mg.\ g^{-1}$ of total increase CDW)	Mn uptake ($mg.\ g^{-1}$ of total increase CDW)
A.34.33%BW+Mn+Mg+EDTA	0.829	3.316	0.522	0.050
B.34.33%BW+Mn+Fe+EDTA	2.747	10.988	1.731	0.165
C.34.33%BW+Mn+Mg+Fe+EDTA	6.810	27.241	4.291	0.409
D.34.33%BW	0.899	3.594	0.566	0.054
E.KC (3.22 g KNO3)	4.891	19.563	3.081	0.293

In the third analysis, growth limitation of *A. obliquus* in BW was determined for the 3 elements (Mg, Mn and Fe) when algae reached the stationary phase (Figure 15). In media B and C, productivities of *A. obliquus* were higher than in medium E (Table 12). In media B and C, growth limitation was observed at Mn concentration of between 0.087-0.114 mg L^{-1}. In medium E, in which *A. obliquus* grew well, growth limitation determined at Fe concentration of 0.082 mg L^{-1}. Growth limitation of magnesium was determined also in medium B of 1.226 mg L^{-1}.

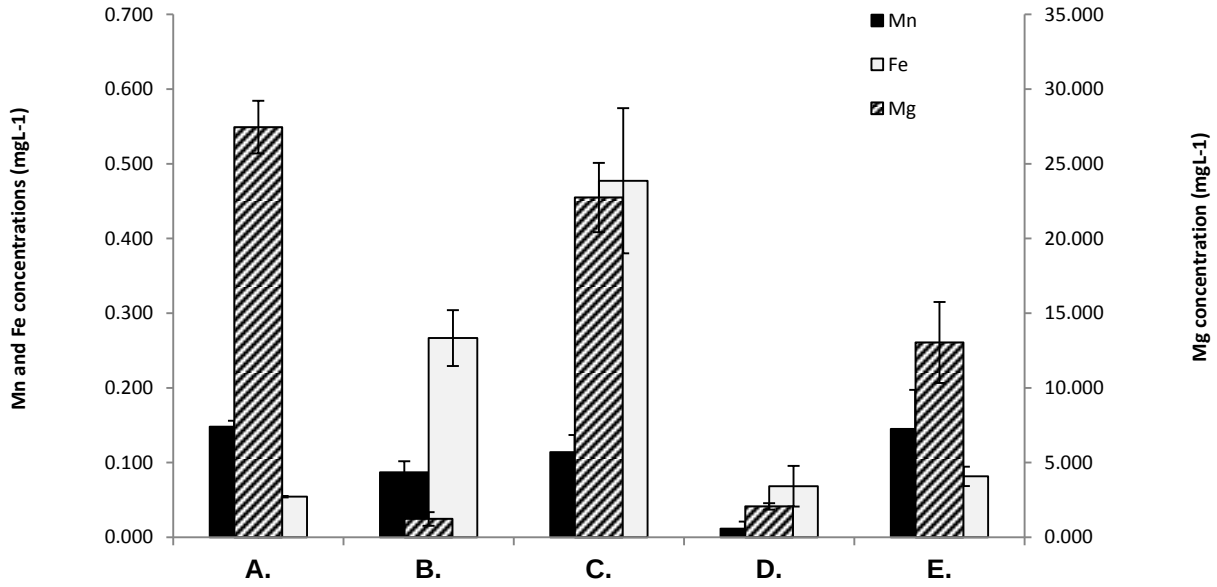


Figure 15. Concentrations of Mg, Mn and Fe at the stationary phases of *A. obliquus* in different composition of BW. Nomenclature according to Table 13. Mean values at the end of experiment were calculated from triplicate ± standard deviation.

3.1.1.3.3 Nutrients removal and uptake of non-limiting elements in black water

With addition of Mn, Mg and Fe in medium C, the percentage of TN removal was significantly 1.19 times higher than in medium B and 1.83 times higher than in medium A (ANOVA F=5515,931, d.f. 4,10, p≤0.05) (Table 14) . However, total N removal in medium E was 1.06 times higher than in medium C. On the other hand, although N uptake per gram CDW in

medium C was 39.5% of medium A and 48.99% of medium B, the total amount of N uptake by cells in medium C (262.32 mg) was highest compared to other BW media (25.4-216.21 mg) (Table 15).

Table 14. Nitrogen removal from different media after 24 days of experiment *

	$N_{day0\_in\ dissolved}$ (mgL^{-1})	$N_{day24\_in\ dissolved}$ (mgL^{-1})	$N_{removal\ (\%)}$
A.34.33%BW+Mn+Mg+EDTA	330.783±9.37	185.917±5.99	43.80±0.24
B.34.33%BW+Mn+Fe+EDTA	322.233±1.26	105.053±4.43	67.40±1.25
C.34.33%BW+Mn+Mg+Fe+EDTA	336.017±4.50	67.060±3.31	80.05±0.72
D.34.33%BW	324.517±0.70	290.483± 0.76	10.49±0.09
E.KC (3.22 g KNO3)	433.700±0.96	66.977±2.69	84.56±0.62

*Values are averages of triplicate with standard deviation

Table 15. Calculated nitrogen uptake into cells from different media after 24 days of experiment*

	CDW increased in 24 days (gL^{-1})	$N_{uptake\ by\ cells}$ ($mg.g^{-1}CDW$)	$N_{uptake\ rate\ by\ cells}$ ($mg.g^{-1}CDW.d^{-1}$)
A.34.33%BW+Mn+Mg+EDTA	0.83 ± 0.009	97.49 ± 1.27	4.06 ± 0.053
B.34.33%BW+Mn+Fe+EDTA	2.75 ±0.051	78.62 ± 4.01	3.28 ± 0.167
C.34.33%BW+Mn+Mg+Fe+EDTA	6.81 ± 0.312	38.52 ± 8.53	1.60 ± 0.355
D.34.33%BW	0.90 ± 0.016	28.22 ± 3.21	1.18 ± 0.134
E.KC (3.22 g KNO3)	4.89 ± 0.191	69.17 ± 2.50	2.88 ± 0.104

*Values are averages of triplicate with standard deviation

In medium C (in which 3 limited elements are added), P removal was significantly 1.35 times higher than in medium B and 1.44 times higher than in medium A (ANOVA F=428.982, d.f. 4,10, p≤0.05) (Table 16). On the other hand, although the lowest P uptake per gram CDW was observed in medium C, the total amount of P uptake by cells in medium C was the highest (13.08 mg) (Table 17). However, in all media compositions the amount of TN and TP uptake by cells was lower than the TN and TP removed from the media. The discrepancy will be discussed later.

Table 16. Phosphorus removal from different media after 24 days of experiment

	$P_{day0\_in\ dissolved}$ (mgL^{-1})	$P_{day24\_in\ dissolved}$ (mgL^{-1})	$P_{removal\ (\%)}$
A.34.33%BW+Mn+Mg+EDTA	20.72±0.202	13.08±0.123	36.84±0.912
B.34.33%BW+Mn+Fe+EDTA	20.81±0.602	12.63±0.611	39.30±1.325
C.34.33%BW+Mn+Mg+Fe+EDTA	27.54±0.493	12.90±0.100	53.15±1.101
D.34.33%BW	22.73±0.473	15.43±0.306	32.09±1.941
E.KC (3.22 g KNO3)	22.03±0.252	8.88±0.126	59.68±0.524

*Values are averages of triplicate with standard deviation

Table 17. Calculated phosphorus uptake into cells from different media after 24 days of experiment

	CDW increased in 24 days (gL^{-1})	$P_{uptake\ by\ cells}$ ($mg.g^{-1}CDW$)	$P_{uptake\ rate\ by\ cells}$ ($mg.g^{-1}CDW.d^{-1}$)
A.34.33%BW+Mn+Mg+EDTA	0.83 ± 0.009	6.65 ± 0.36	0.28 ± 0.01
B.34.33%BW+Mn+Fe+EDTA	2.75 ±0.051	2.81 ± 0.13	0.12 ± 0.01
C.34.33%BW+Mn+Mg+Fe+EDTA	6.81 ± 0.312	1.92 ± 0.10	0.08 ± 0.00
D.34.33%BW	0.90 ± 0.016	7.25 ± 0.14	0.30 ± 0.01
E.KC (3.22 g KNO3)	4.89 ± 0.191	2.61 ± 0.12	0.08 ± 0.06

*Values are averages of triplicate with standard deviation

To observe nutrient accumulation in the cells of *A. obliquus*, carbon, nitrogen, and phosphorus ratios to CDW were calculated (Figure 16).The highest carbon (66.23%) and the lowest phosphorus accumulation (0.19%) in cells was observed in medium C, in which optimal growth of *A. obliquus* in black water media occurred. On the other hand, high phosphorus accumulation of 0.67 and 0.73%, respectively, was observed in media A and D, where growth was limited.

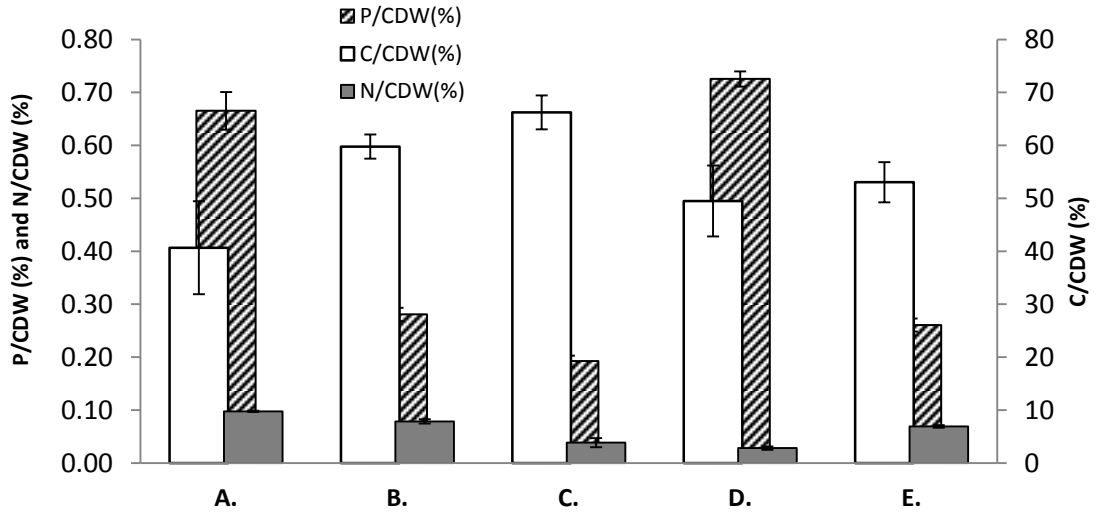


Figure 16. Ratio of carbon, nitrogen, and phosphorus to cell dry weight of *A. obliquus* in various media compositions. Mean values are averaged from triplicate ± standard deviation.

3.1.1.3.4 Pigment content in algal cells cultivated in black water

The 5 peaks presented in the chromatograms of *A. obliquus* cultivated in different media compositions indicated that the same pigments always occurred (Figure 17). Similar chromatograms were shown by all replicates (Appendix 1).

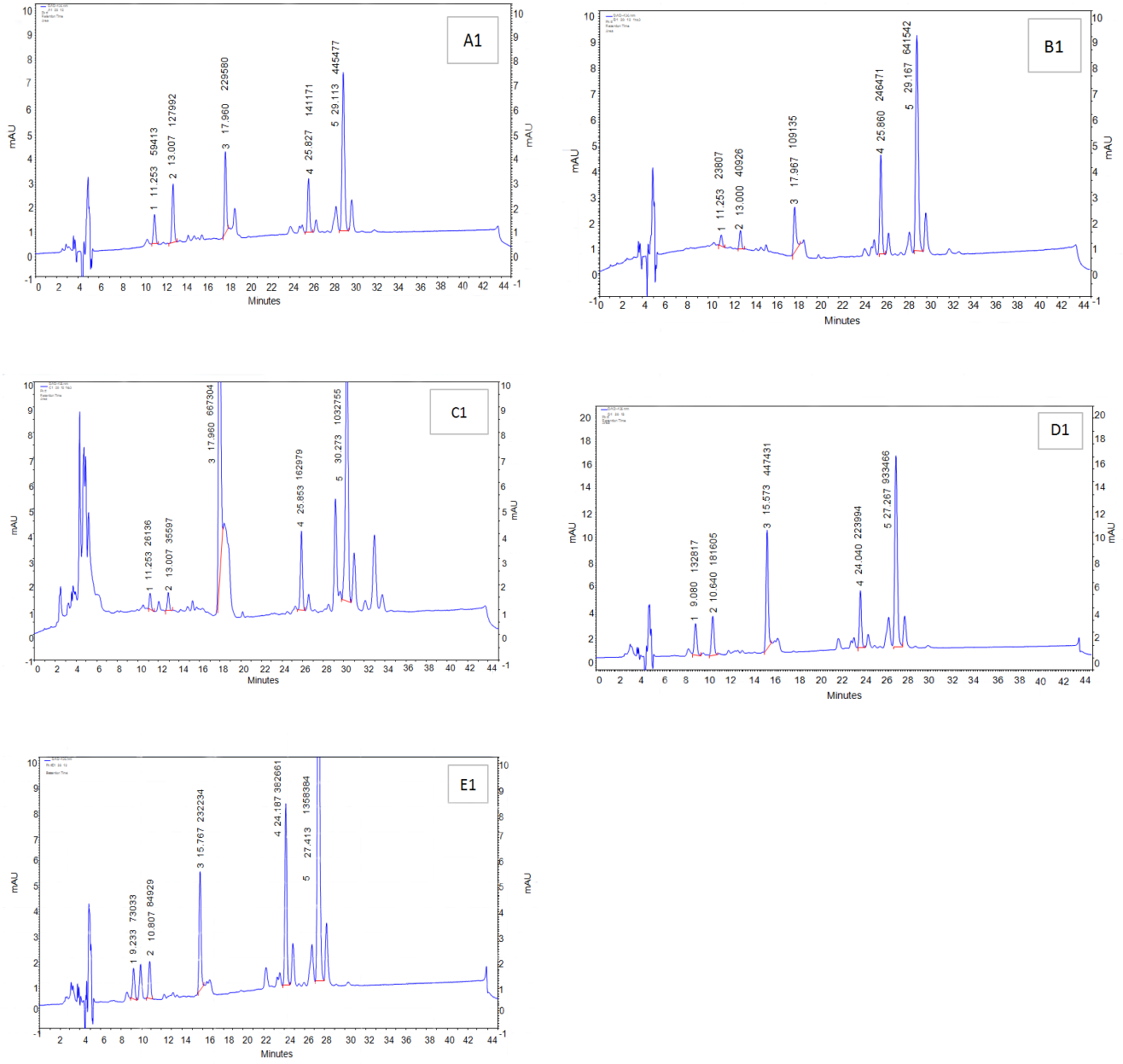
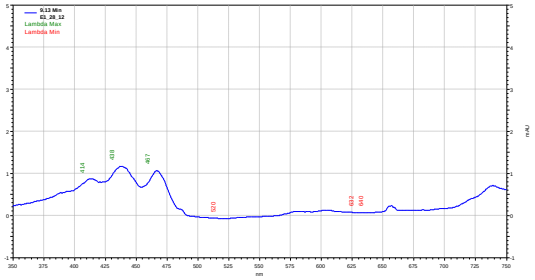


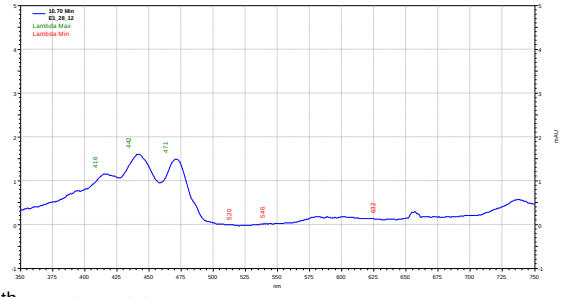
Figure 17. Chromatograms of pigments produced by *A.obliquus* in different media composition: A1.BW+Mn+Mg; B1.BW+Mn+Fe; C1.BW+Mn+Mg+Fe D1.BW; E1.KC

Spectral analysis of pigments, detected with a single wavelength at 436 nm, identified these 5 pigments as neoxanthin, violaxanthin, lutein, chlorophyll b and chlorophyll a (Figure 18).

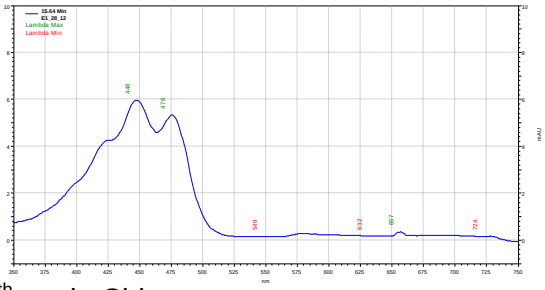
1st peak: Neoxanthin



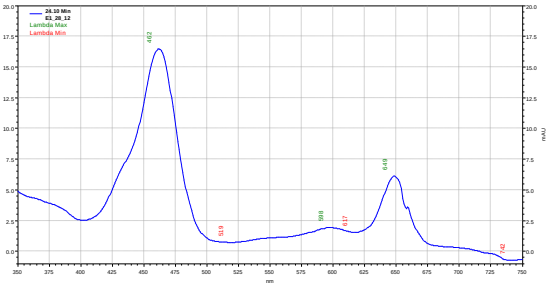
2nd peak: Violaxanthin



3rd peak: Lutein



4th peak: Chl b



5th peak: Chl a

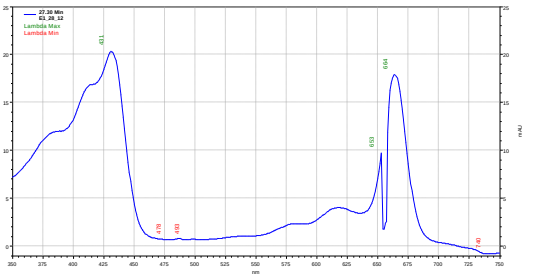


Figure 18. Spectrums of 5 different pigments observed from the chromatogram in Figure 8 above

Comparison between different BW media indicated that the lowest pigment content in *A. obliquus* was observed in medium C (Table **18**). Although biomass productivity of *A. obliquus* was 1.2 times higher, carotenoids content in medium C was only 5.67%, 6.27%, and 82.9% for neoxanthin, violaxanthin, and lutein, compared with the results of medium E (control). Likewise, the chlorophyll a concentration of cells in medium C was only 9% of the chlorophyll a concentration of cells in medium E (Figure 19).

Table 18. Absolute pigment concentrations of *A. obliquus* cultivated in BW (ng/µg DW)

	Neoxanthin	Violaxanthin	Lutein	Chl b	Chl a
A.34.33%BW+Mn+Mg+EDTA	0.307±0.015	0.520±0.026	1.714±0.06	2.184±0.115	5.001±0.188
B.34.33%BW+Mn+Fe+EDTA	0.032±0.001	0.043±0.001	0.239±0.009	1.043±0.043	1.646±0.082
C.34.33%BW+Mn+Mg+Fe+EDTA	0.016±0.000	0.017±0.001	0.937±0.026	0.242±0.004	0.951±0.046
D.34.33%BW	0.539±0.024	0.581±0.009	2.171±0.05	3.069±0.128	7.702±0.147
E.KC (3.22 g KNO3)	0.282±0.015	0.271±0.012	1.130±0.05	4.956±0.2	10.540±0.259

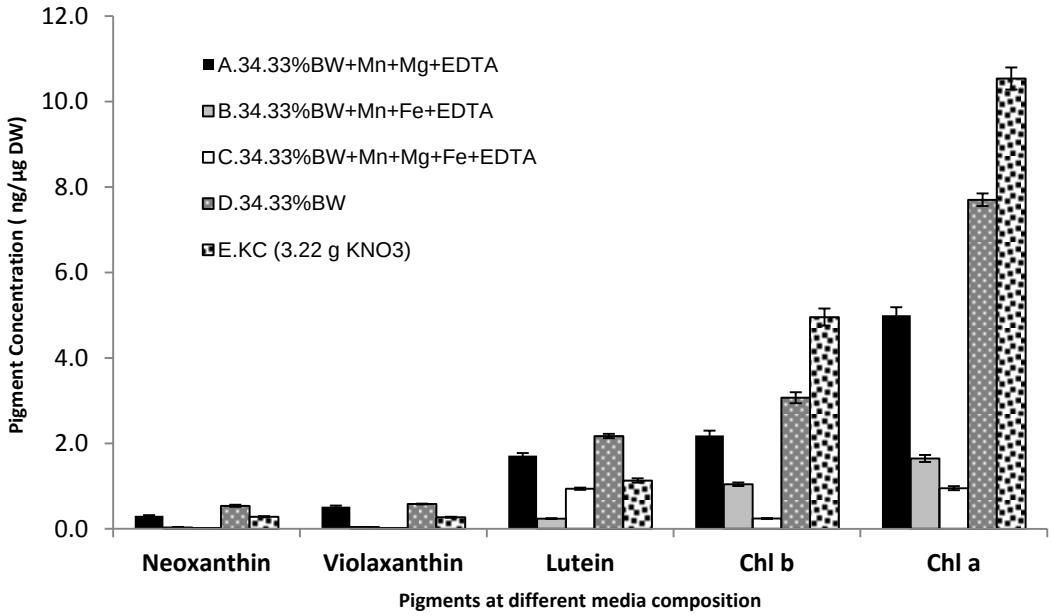


Figure 19. Pigment concentration in cells of *A. obliquus* in Black Water

3.1.1.3.5 Transmission Electron Microphotographs

During the twenty-four days of the experiment, the development of resting spore cells was initiated. Resting spore cells were indicated with wavy cell walls and darkening of the cytosol. The amount of resting spore cells differed between different culture media (Table 19). Among 40-45 cells were examined in every medium composition (Appendix 2), the lowest number of resting spore cells was observed in medium E, in which *A. obliquus* grew without BW (Figure 24, nomenclature of figures according to the Table 19). On the contrary, more than 65% of resting spore cells was observed when *A. obliquus* grew in BW media (Figure 20 - Figure 23, nomenclature of figures according to Table 19).

Reduction of thylakoid membranes was observed in most of *A. obliquus* cells cultivated in different compositions of BW media, and starch and/or lipid production was promoted. In contrast, most *A. obliquus* cells grown in control medium (KC) showed complete thylakoid membrane structures but less starch and no lipid production (Figure 24). Interestingly, accumulation of lipids occurred in resting spore cells in medium B, in which Mg was the limiting element (Figure 21).

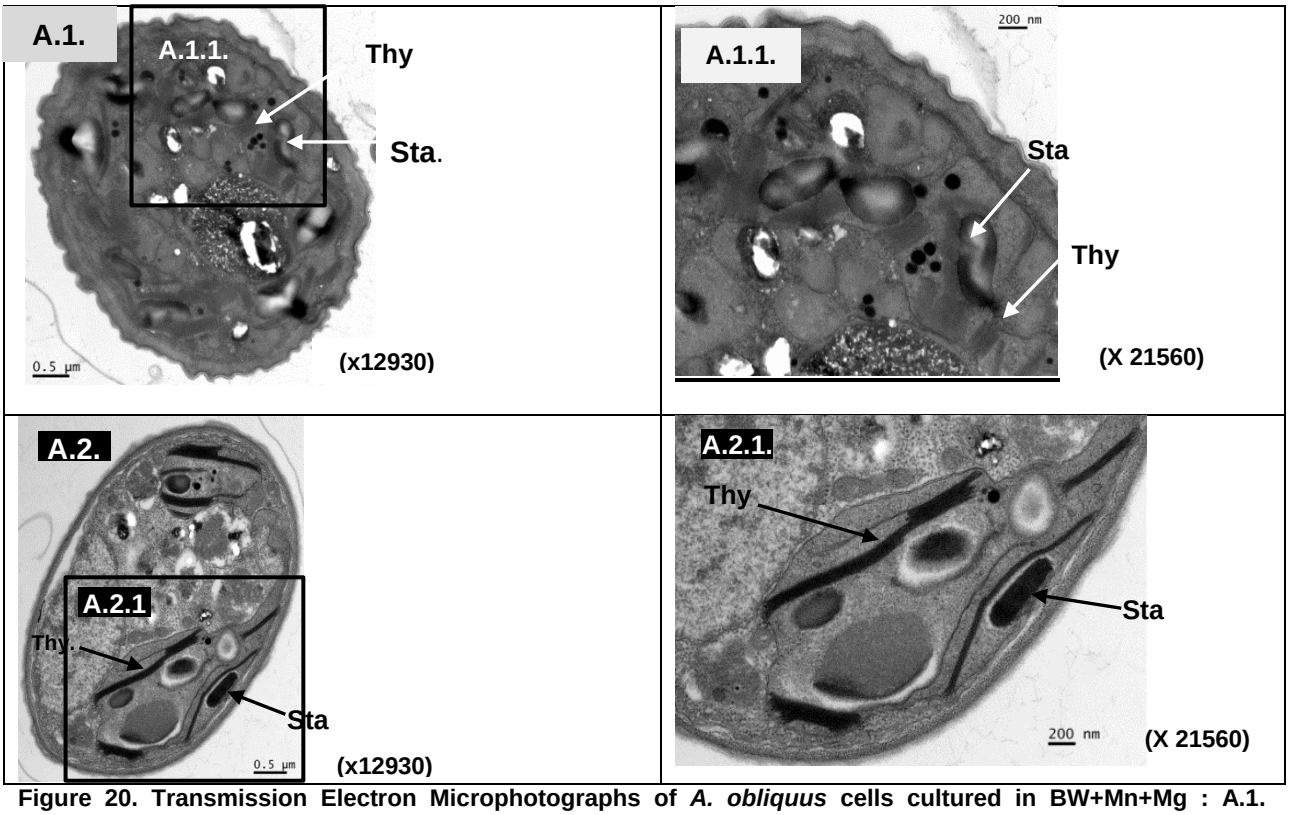


Figure 20. Transmission Electron Microphotographs of *A. obliquus* cells cultured in BW+Mn+Mg : A.1. Resting spore cells; A.2. Vegetative cells. Thy: thylakoid; Sta: starch. Cellular details within the quadrat of the left figure are shown in higher resolution in the following right figure.

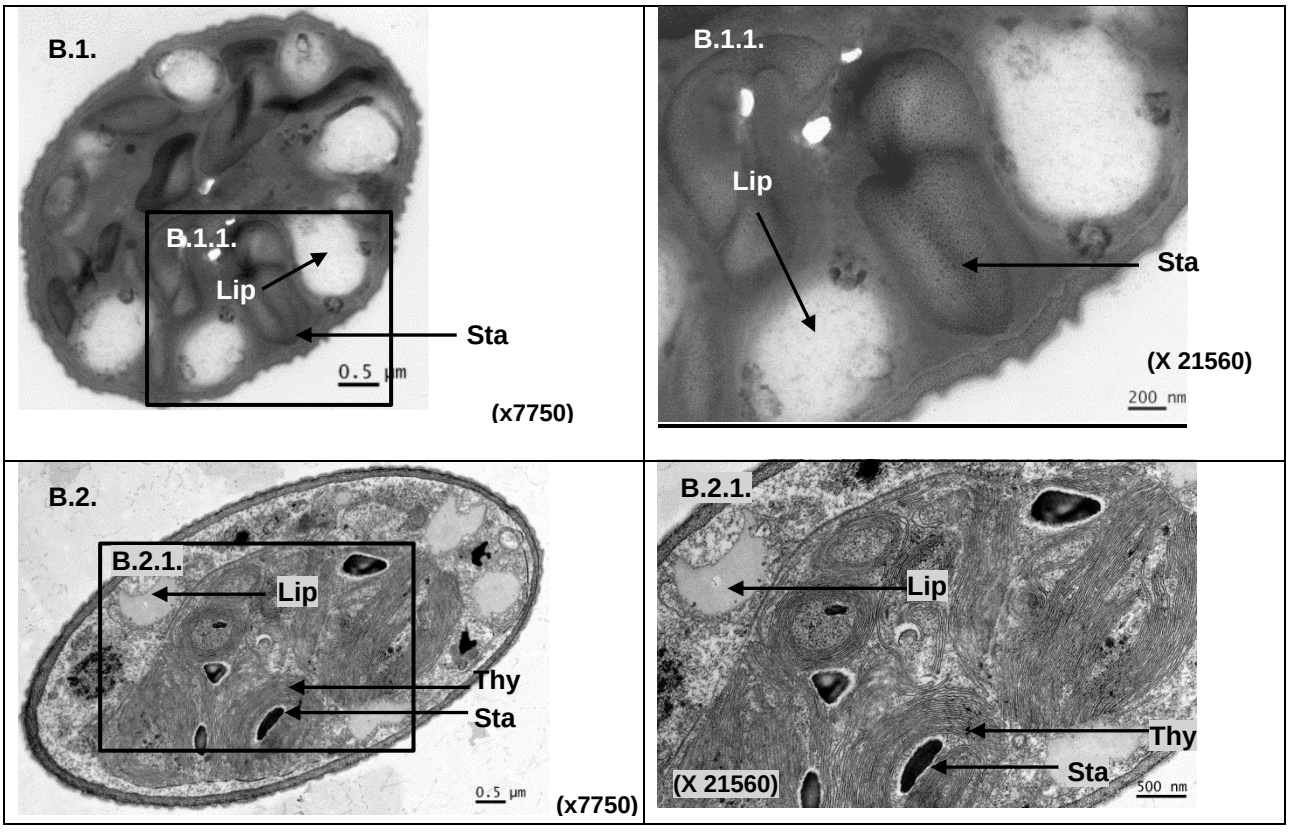


Figure 21. Transmission Electrone Microphotographs of *A. obliquus* cultured in BW+Mn+Fe : B.1. Resting spore cells and B.2. Vegetative cells. Thy: thylakoid; Sta: starch; Lip: lipid. Cellular details within the quadrat of the left figure are shown in higher resolution in the following right figure.

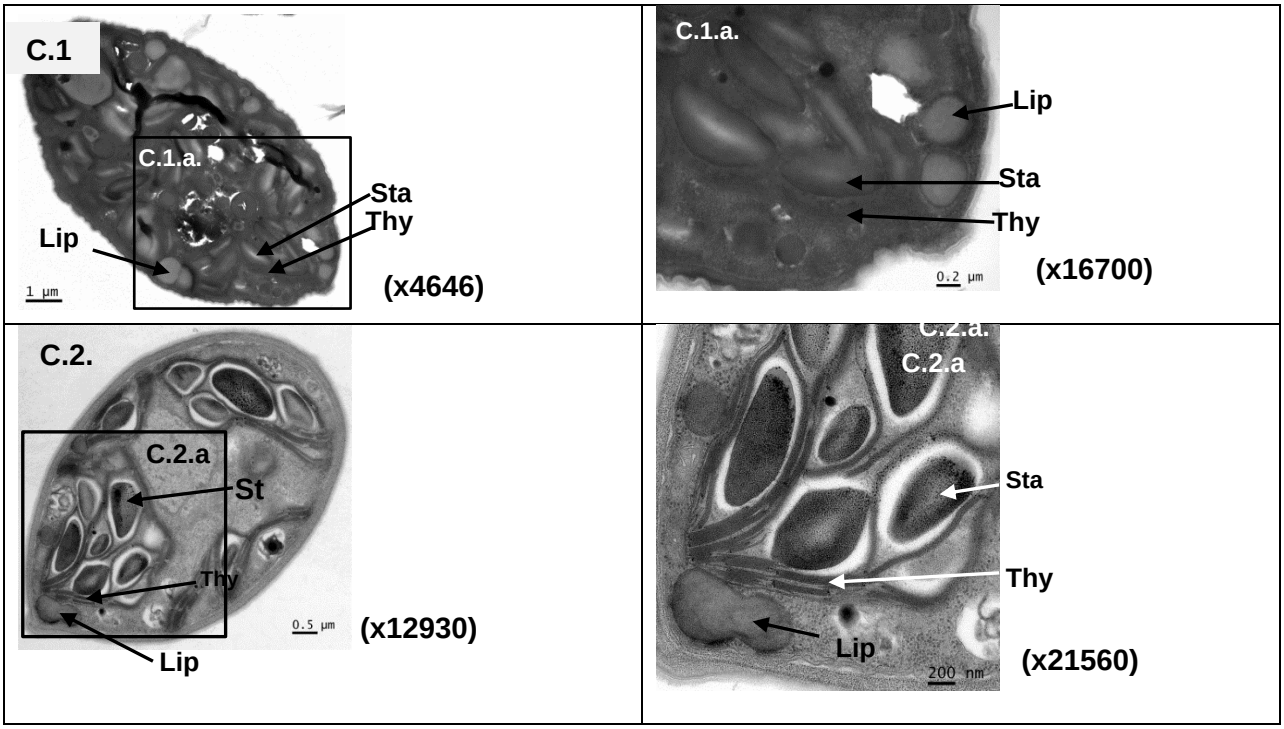


Figure 22. Transmission Electron Microphotographs of *A. obliquus* cultured in BW+Mn+Mg+Fe : C.1. Resting spore cells and C.2. Vegetative cells. Thy: thylakoid; Sta: starch; Lip: lipid. Cellular details within the quadrat of the left figure are shown in higher resolution in the following right figure.

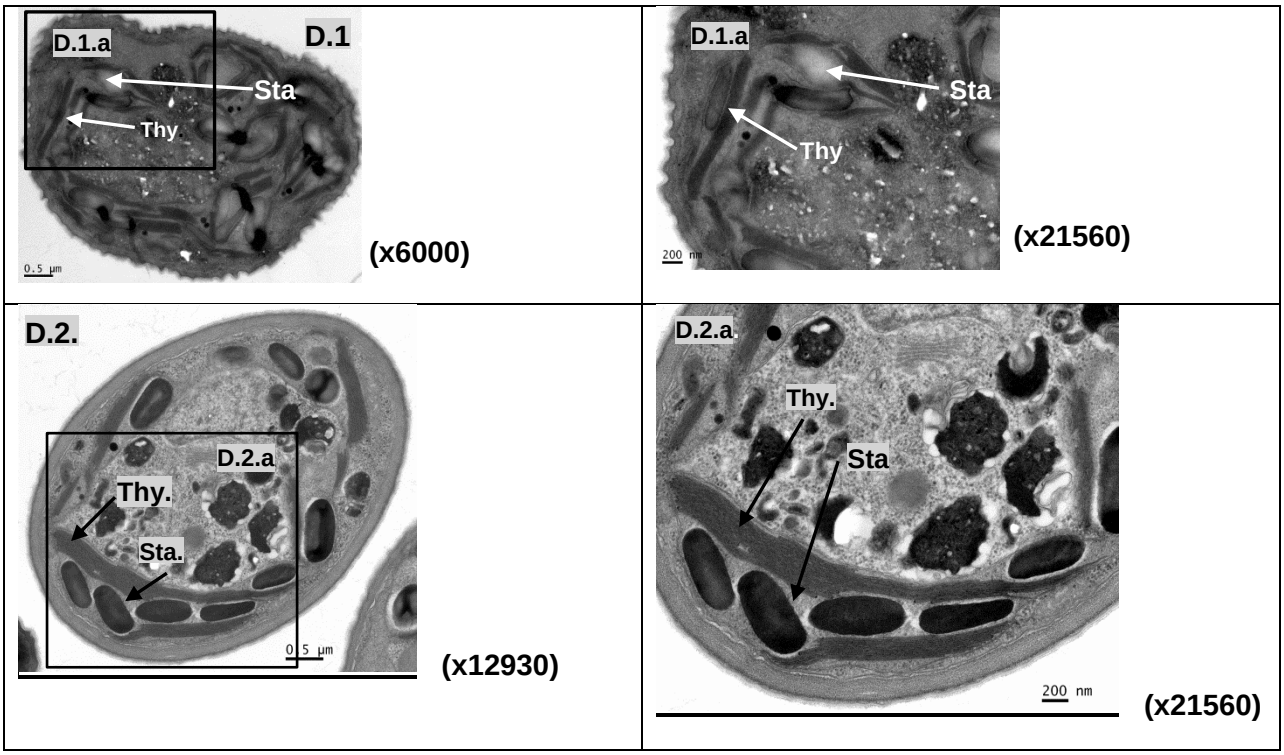


Figure 23. Transmission Electrone Microphotographs of *A. obliquus* cultured in BW: D.1. Resting spore cells and D.2. Vegetative cells. Thy: thylakoid; Sta: starch. Cellular details within the quadrat of the left figure are shown in higher resolution in the following right figure.

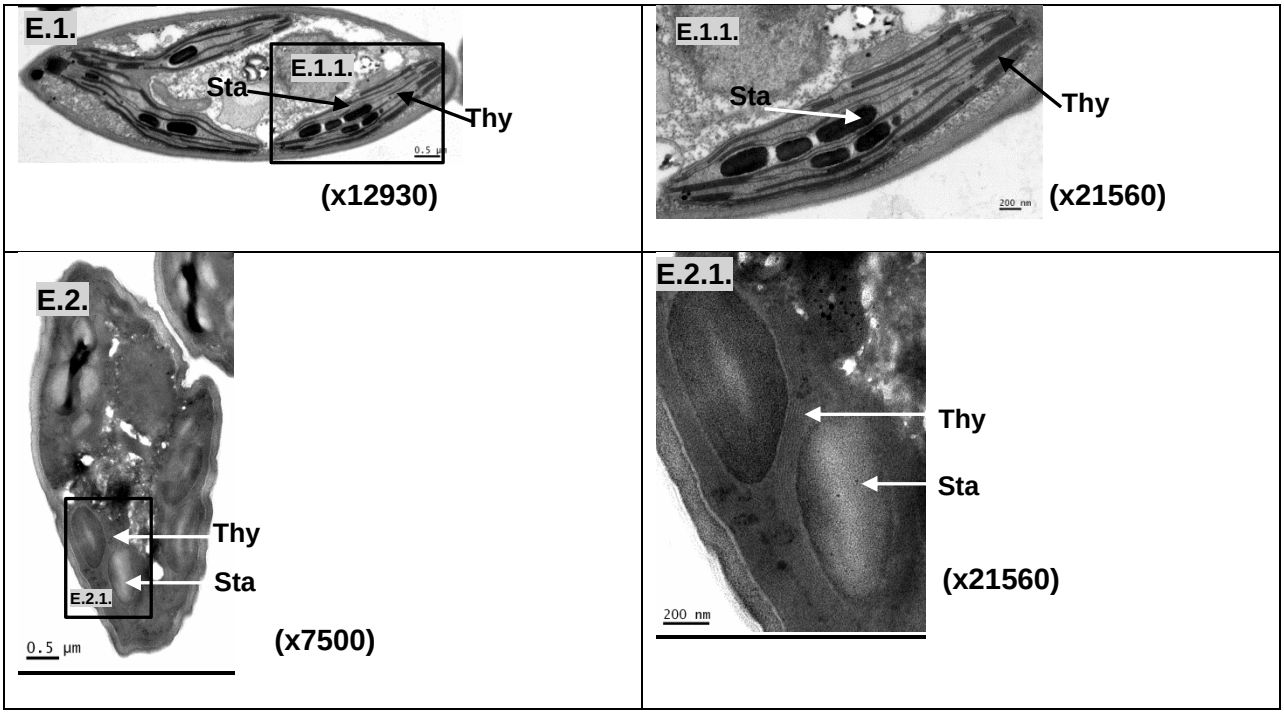


Figure 24. Transmission Electron Microphotographs of *A. obliquus* cultured in KC media: E.1. Vegetative cells and E.2. Resting spore cells. Thy: thylakoid; Sta: starch. Cellular details within the quadrat of the left figure are shown in higher resolution in the following right figure.

Table 19. Comparison of resting spore cells and vegetative cells of *A. obliquus* in different media

	Resting spore cell (%)	Vegetative cells (%)
A.34.33%BW+Mn+Mg+EDTA	90	10
B.34.33%BW+Mn+Fe+EDTA	90	10
C.34.33%BW+Mn+Mg+Fe+EDTA	70	30
D.34.33%BW	69	31
E.KC (3.22 g KNO_3)	34	66

3.1.2 Cultivation of algae in BIQ wastewater

3.1.2.1 Characteristics of BIQ wastewater

A mixture process of black water and gray water reduced the concentration of all major elements concentrated in the black water. Compared to BW (Table 9), the concentrations of major elements in the BIQ wastewater used in this experiment (Table 20) were only 12.7%, 14%, 12.8% and 23.8%, for Total Carbon (TC), Total Organic Carbon (TOC), Total N (TN) and Total P (TP), respectively.

As expected, after anaerobic digestion of this BIQ wastewater, the TN and TP concentration remained about the same (97.73 and 95.7%, respectively). However, only 76.83% of TP remained after the filtration process (Table 20).

Table 20. Characteristics of BIQ wastewater

	BIQ wastewater before anaerobic digestion (mg/L)	BIQ wastewater after anaerobic digestion (mg/L)	BIQ wastewater after anaerobic digestion and filtration by 0.1 µm pore size (mg/L)
TC	859	698	342
TOC	813	480	137
TN	220	215	193
NH_4-N	180	182	181
NO_3-N	0.78	< 0,5	< 0,5
TP	41.8	40.0	9.37
PO_4-P	13.7	12.0	9.22

Element concentration observed in the BIQ wastewater was lower than that measured in BW (Table 11). Concentration of elements occurred in the BIQ wastewater (Table 21), such as sodium, manganese, iron, zinc and boron were only 29.47%, 40%, 18.33%, 37.5%, and 11.72%, respectively of those in BW.

Table 21. Concentration of elements in the 1st BIQ wastewater

	BIQ_wastewater (mgL^{-1})
K	64.6
Ca	29.84
Mn	0.008
Fe	0.022
Zn	0.015
B	0.211
Mg	10.404

3.1.2.2 Algal growth in BIQ wastewater

Based on the results above, Mn, Mg and Fe were added to test elemental limitations in the BIQ wastewater to support the optimal growth of *A. obliquus*. The magnesium, manganese and iron were supplemented at concentration referring to the KC medium.

Without additional elements, *A. obliquus* reached stationary phase faster when growing in BIQ wastewater (Figure 25). Growth was initiated in BIQ wastewater after elements were added at day-23 which clearly showed the element limitation of BIQ wastewater for *A.obliquus* growth.

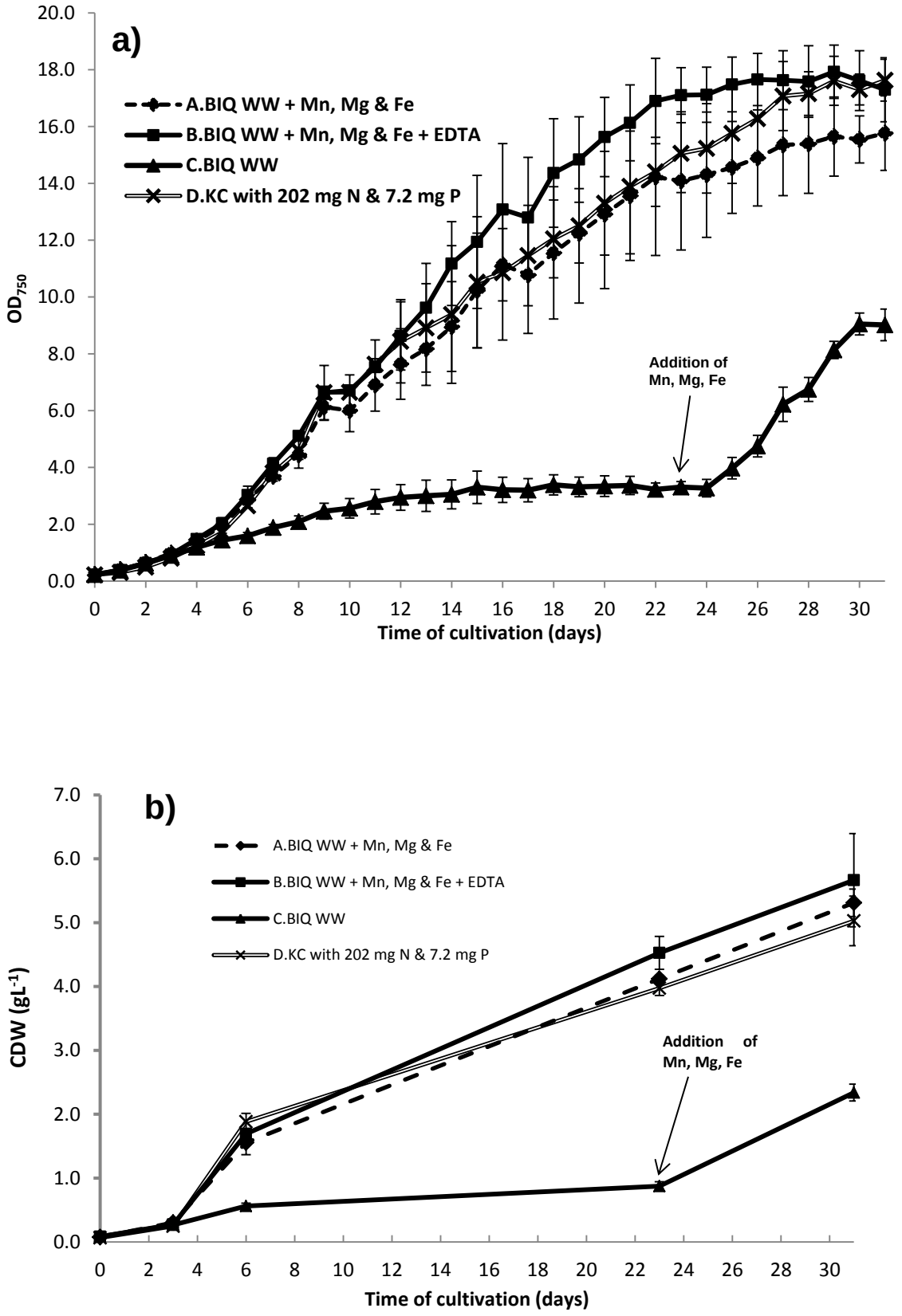


Figure 25. Growth of *A. obliquus* in different compositions of BIQ wastewater and in KC, based on: a). Optical Density; b). Cell dry weight. Mean values were calculated from triplicate ± standard deviation.

The highest productivity was achieved when *A. obliquus* grew in the BIQ wastewater with additional Mn, Mg, Fe, and EDTA (Table 22). Productivity in medium B with additional EDTA was significantly 1.12 times higher than in medium A (without EDTA) (ANOVA F=401,637, d.f.=3, 8, p≤ 0.05). However, no significant difference was observed in the specific growth rate of *A. obliquus* between medium A and medium B and between medium B and medium D.

Table 22. Productivity and specific growth rate of *A. obliquus* in different compositions of BIQ wastewater (media A, B, C) and in a control medium (D)

Culture conditions	Cell Dry Weight (CDW) at the start of exponential phase ($g\ L^{-1}$)	(CDW) at the end of exponential phase ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ d^{-1}$)	Specific Growth Rate in CDW (d^{-1})
A.BIQ WW + Mn, Mg & Fe	0.302 ± 0.023	4.121 ± 0.052	0.191 ± 0.004	0.131± 0.004
B.BIQ WW + Mn, Mg & Fe + EDTA	0.273 ± 0.023	4.527 ± 0.258	0.213 ± 0.012	0.140 ± 0.003
C.BIQ WW	0.266 ± 0.035	0.874 ± 0.069	0.030 ± 0.005	0.060 ± 0.009
D.KC with 202 mg N & 7.2 mg P	0.245 ± 0.020	3.975 ±0.115	0.187 ± 0.005	0.139 ± 0.004

As found in the BW experiment, photosynthetic activity (Fv/Fm values) of *A. obliquus* on the first day of inoculation was very low (0.437- 0.496). Except in medium C, an increase of Fv/Fm values after day-3 was observed in media A, B and D (Figure 26). Addition of elements of Mg, Mn and Fe at day-23 initiated a rise of photosynthetic activity of *A. obliquus* in medium C (0.413-0.617) to the same level to the Fv/Fm values of *A. obliquus* in the other media.

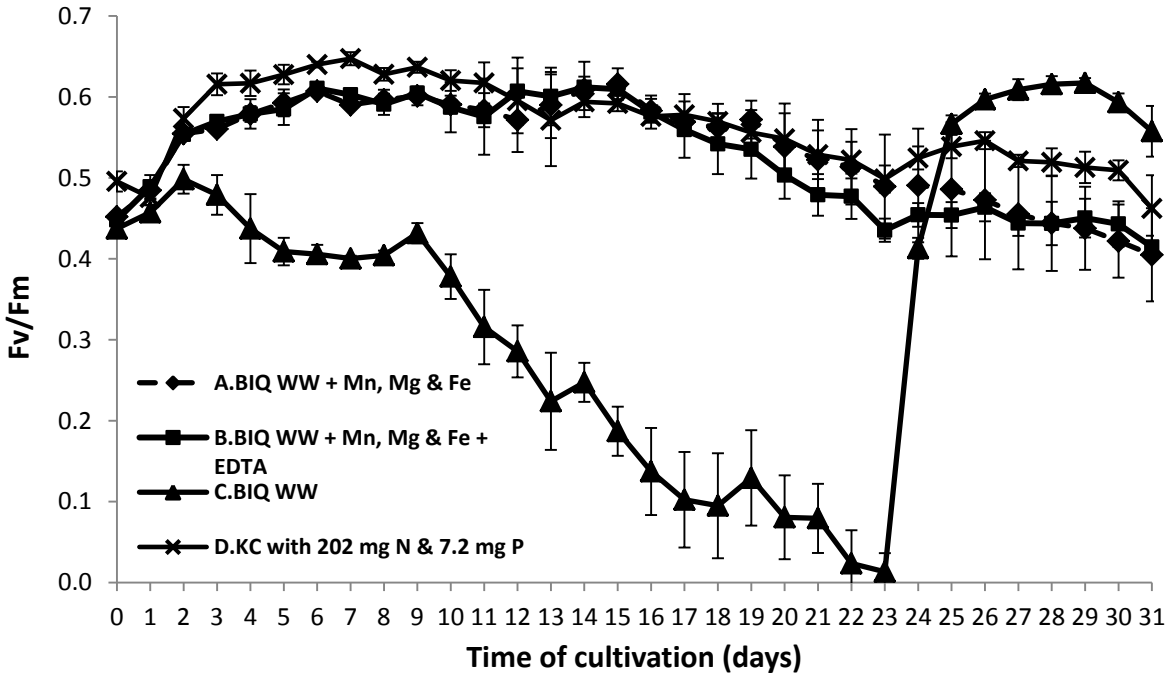
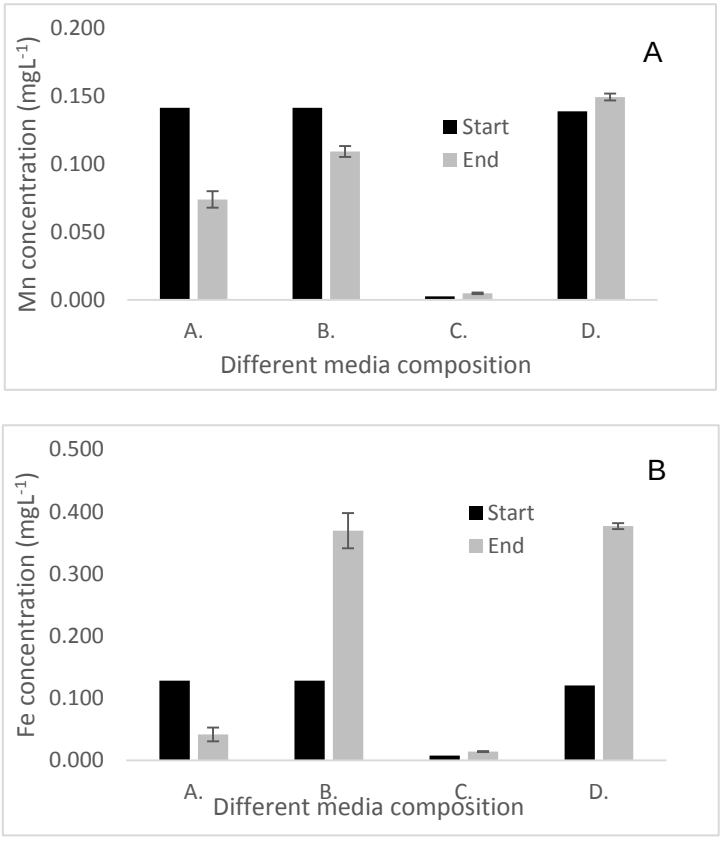


Figure 26. Photosynthetic efficiency of *A. obliquus* during their growth in three different BIQ wastewater compositions and in a KC medium. Mean values were calculated from triplicate ± standard deviation.

3.1.2.3 Element limitation in the BIQ wastewater

As had been analyzed in the experiment with BW, in the first analysis, growth limitation of *A. obliquus* in BIQ wastewater was calculated based on demand for 3 elements of interest (Mn, Mg and Fe). Based on the element concentration of the media at the beginning and end of the experiment, especially in medium B (in which *A. obliquus* grew optimally), only removal of manganese of 0.032 mg L^{-1} was observed (Figure 27 A). At the end of the experiment B, iron and magnesium were even 2.88 and 1.17 times higher, respectively, than at the beginning (Figure 27 B and C). The highest Mn (0.067 mg L^{-1}) and Fe (0.086 mg L^{-1}) removal was observed in medium A, where no EDTA was added (Figure 27 A and B).



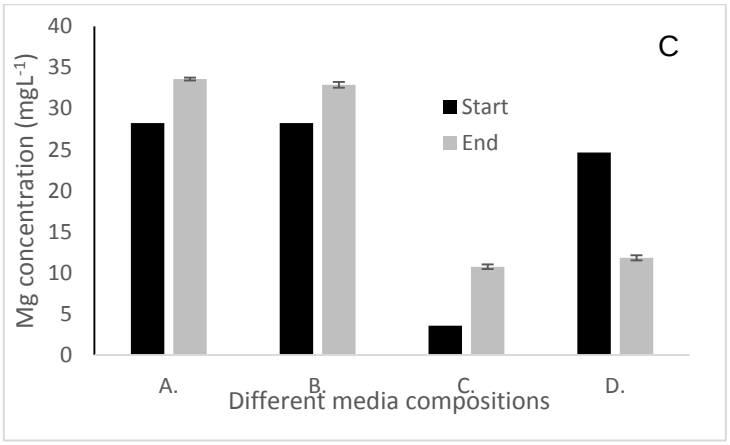


Figure 27. Concentration of: A: Mn; B: Fe and C: Mg in different compositions of BIQ wastewater. Mean values at the end of experiment were calculated from triplicate ± standard deviation.

In the second analysis, elements needed to support *A. obliquus* growth was calculated based on the percentage of CDW, referring to Elsayed et al. (2016) and Hecky and Kilham (1988) . Calculating the percentage of elements in the cell dry weight, about 22.33, 3.52 and 0.33 mg g^{-1}CDW, respectively for Mg, Fe and Mn was uptaken in medium B (Table 23), in which the highest productivity of *A. obliquus* was observed. At the beginning of the experiment Mg, Fe and Mn concentrations of 28.22, 0.128 and 0.141 mg L^{-1}, respectively, were only supplemented. Thus, only Mg was sufficiently added and 5.89 mg L^{-1} has probably remained in the medium.

Table 23. Magnesium, iron and manganese uptake concentrations in BIQ wastewater media based on the increased of CDW (modified from Elsayed et al. 2016; Hecky and Kilham 1988).

	Increased CDW (gL^{-1})	Mg uptake (mg. g^{-1} increased CDW)	Fe uptake (mg. g^{-1} increased CDW)	Mn uptake (mg. g^{-1} increased CDW)
A.BIQ WW + Mn, Mg & Fe	5.24	20.95	3.30	0.31
B.BIQ WW + Mn, Mg & Fe + EDTA	5.58	22.33	3.52	0.33
C.BIQ WW	2.26	9.02	1.42	0.14
D.KC with 202 mg N & 7.2 mg P	4.96	19.86	3.13	0.30

In the third analysis, possible growth limitation by Fe, Mn and Mg were examined at the stationary phase (Figure 28). From medium A, growth limitation was observed at Fe concentration of 0.042 mg L^{-1}. However, in the BW experiment Fe limitation already occurred below 0.08 mg L^{-1}. From medium B, it can be deduced that growth was limited by an Mn concentration of 0.109 mg L^{-1}. This value is similar to that found in the BW experiment. In this experiment, Mg limitation cannot be inferred. If magnesium limitation is inferred from media D, then the value is dubious, because with a similar amount of Mg in medium C, no growth of *A. obliquus* was observed.

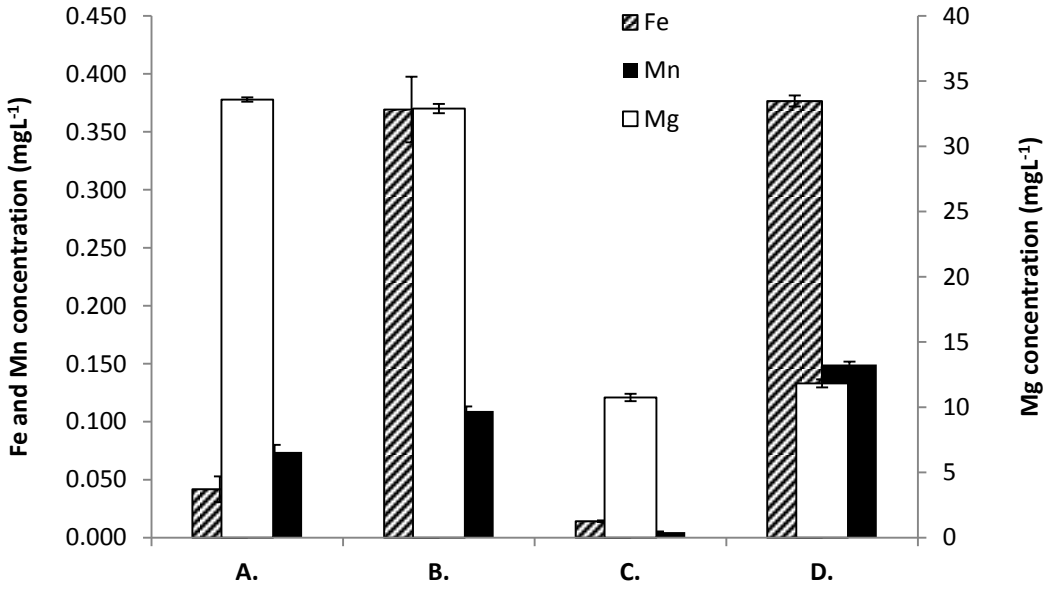


Figure 28. Concentration of Mg, Mn and Fe at the stationary phases of *A. obliquus* in different composition of BIQ wastewater and in a KC medium. Mean values were calculated from triplicate ± standard deviation. Nomenclature according to Table 23.

3.1.2.4 Nutrient removal and uptake of non-limiting elements in the BIQ wastewater

After 23 days of the experiment, the highest total N removal reached 67.7% in medium A. Nevertheless, no significant difference was found between total N removal in medium A and medium B (67.4%) (ANOVA, F=1269,341, d.f. 3,8, p≥0.05) (Table **24**). With BIQ wastewater as nutrient sources (in medium A), the percentage of total N removal was 1.045 times significantly higher than in the control (medium D) (ANOVA, F=1269.341, d.f. 3,8, p≤0.05). With addition of Mn, Mg, and Fe, N uptake per gram of CDW in medium A was 1.6 times higher than that in medium C and 1.09 times higher than in medium B (**Table 25**). However, among BIQ media, the highest amount of total N uptake by the cells (124.28 mg N) was observed in medium B.

Table 24. Nitrogen removal from different media composition of BIQ wastewater and control (modified KC) cultivated with *A. obliquus*

	Nday0_in dissolved (mgL^{-1})	Nday23_in dissolved (mgL^{-1})	Nremoval in 23 days (mgL^{-1})	Nremoval (%)
A.BIQ WW + Mn, Mg & Fe	187.95±1.12	60.77±0.25	127.19±1.37	67.67±0.33
B.BIQ WW + Mn, Mg & Fe + EDTA	186.57±1.08	60.83±1.06	125.74±0.17	67.40±0.38
C.BIQ WW	191.05±1.77	99.20±0.75	91.85±1.05	48.08±0.14
D.KC with 202 mg N & 7.2 mg P	208.12±0.25	73.37±1.58	134.76±1.51	64.75±0.74

Table 25. Calculation of nitrogen uptake into cells from different media compositions of BIQ wastewater and control (modified KC) medium

	CDW increased in 23 days (gL^{-1})	Nuptake by cells ($mg.g^{-1}CDW$)	Nuptake rate by cells ($mg.g^{-1}CDW.d^{-1}$)
A.BIQ WW + Mn, Mg & Fe	4.05 ± 0.06	30.47 ± 0.65	1.32 ± 0.03
B.BIQ WW + Mn, Mg & Fe + EDTA	4.44 ± 0.25	27.99 ± 1.68	1.22 ± 0.07
C.BIQ WW	0.79 ± 0.07	19.06 ± 2.52	0.83 ± 0.11
D.KC with 202 mg N & 7.2 mg P	3.91 ± 0.11	33.55 ± 1.33	1.46 ± 0.06

Compared with the control medium (medium D), the percentage of phosphorus (P) removal in the BIQ wastewater was only 91.22-91.28% (Table 26). There was no statistically significant difference between P removal in medium A and medium B (ANOVA F=1078.062, d.f. 3,8, p≥0.05). Hence, additional supply of EDTA had no influence on the percentage of P removal in the BIQ wastewater media. However, the phosphorus uptake per gram CDW of *A. obliquus* in medium B was 1.18 times higher than that in medium A (Table 27). Likewise, the total amount of P uptake by the cells in medium B (4.17 mg P) was higher than in medium A (3.24 mg P).

Table 26. Phosphorus removal from different compositions of BIQ wastewater cultivated with *A. obliquus*

	$P_{day0\_in\ dissolved}$ (mgL-1)	$P_{day23\_in\ dissolved}$ (mgL-1)	$P_{removal}$ (mgL-1)	$P_{removal\ (\%)}$
A.BIQ WW + Mn, Mg & Fe	6.737±0.152	1.977±0.025	4.76±0.16	70.647±0.843
B.BIQ WW + Mn, Mg & Fe + EDTA	6.667±0.059	1.96±0.053	4.707±0.021	70.603±0.562
C.BIQ WW	6.61±0.095	4.01±0.036	2.6±0.13	39.321±1.402
D.KC with 202 mg N & 7.2 mg P	8.597±0.106	1.943±0.055	6.653±0.081	77.395±0.504

Table 27. Calculation of phosphorus uptake from different compositions of BIQ wastewater cultivated with *A. obliquus*

	CDW increased in 23 days (gL^{-1})	Puptake by cells ($mg.g^{-1}CDW$)	Puptake rate by cells ($mg.g^{-1}CDW.d^{-1}$)
A.BIQ WW + Mn, Mg & Fe	4.05 ± 0.06	0.80 ± 0.04	0.035 ± 0.00
B.BIQ WW + Mn, Mg & Fe + EDTA	4.44 ± 0.25	0.94 ± 0.05	0.041 ± 0.00
C.BIQ WW	0.79 ± 0.07	1.62 ± 0.08	0.071 ± 0.00
D.KC with 202 mg N & 7.2 mg P	3.91 ± 0.11	1.65 ± 0.04	0.072 ± 0.00

3.1.2.5 Pigment content in algal cells cultivated in BIQ wastewater

In this experiment, pigments of *A. obliquus* were collected from all media on the same day (day-31). *A. obliquus* in media A, B and D was already in the stationary phase, while those from medium C was still in the exponential phase. This condition occurred, while unexpected growth still occurred after the addition of elements at day-23. In this case, the results from medium C are not comparable with those from media A, B and D.

As in BW media, neoxanthin, violaxanthin, lutein, chlorophyll b and chlorophyll a were produced by *A. obliquus* in BIQ media (Figure 29). Similar chromatograms were shown by all replicates (Appendix 3). Although similar biomass production was visible in media A, B and D, the maximum amounts of carotenoids produced by *A. obliquus* in the BIQ media were only 43.75%, 52.94%, and 78.12%, for neoxanthin, violaxanthin and lutein, respectively, of those produced in medium D (KC medium) (Table 28).

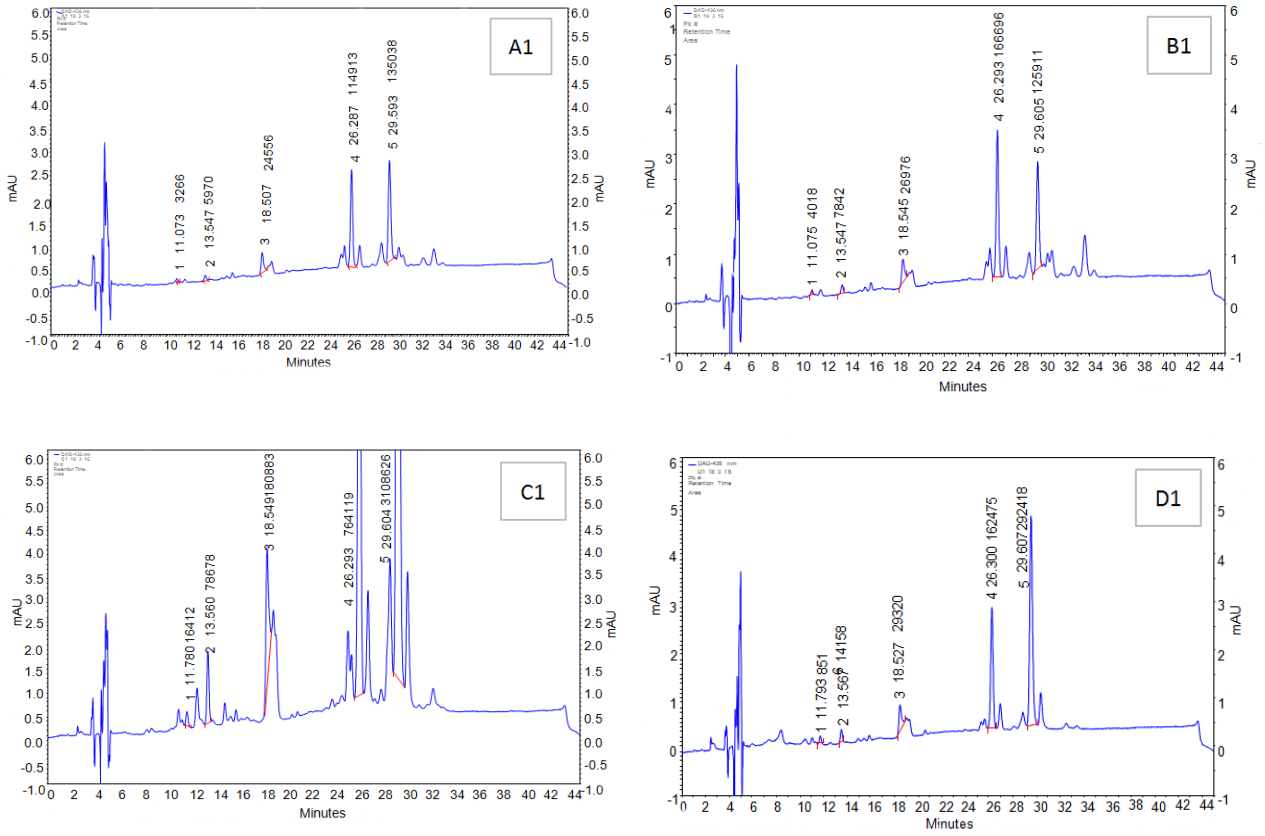


Figure 29. Chromatograms of pigment produced by *A. obliquus* in different media compositions: A1: BIQ wastewater (WW)+Mn, Mg and Fe; B1: BIQ WW +Mn, Mg, Fe plus EDTA; C1: BIQ WW; D1: Control medium (KC)

Similar indication of lower chlorophyll a production in BW than in control medium was also observed in BIQ wastewater media. Compared to that found in medium D, lower productivity of chlorophyll a (37-41%) was observed in BIQ wastewater media (media A and B) (Table 28), although no significant difference in growth rate was observed (Table 22). The concentration of chlorophyll a in BIQ wastewater without EDTA had no significantly statistical difference with chlorophyll a concentration in BIQ wastewater with additional EDTA (Figure 30) (ANOVA F=397.992, d.f. 2,6, p≥0.05). The chlorophyll a concentration of *A. obliquus* in medium C was more than 10 times higher than that in media A, B and D. However, the result of medium C

cannot be compared with other media because the pigment content of *A. obliquus* in medium C was not measured in the same stationary phase as in media A, B and D.

Table 28. Pigment concentrations of *A. obliquus* cultivated in different compositions of BIQ wastewater media and in a control medium (KC) (ng/µg DW).

	Neo	Viola	Lutein	Chl b	Chl a
A.BIQ WW + Mn, Mg & Fe	0.006±0.000	0.008±0.000	0.047±0.001	0.406±0.014	0.277±0.007
B.BIQ WW + Mn, Mg & Fe + EDTA	0.007±0.000	0.009±0.000	0.050±0.002	0.559±0.029	0.249±0.004
C.BIQ	0.031±0.001	0.083±0.003	0.357±0.017	3.195±0.171	7.669±0.242
D.KC with 202 mg N & 7.2 mg P	0.016±0.000	0.017±0.000	0.064±0.002	0.680±0.006	0.678±0.035

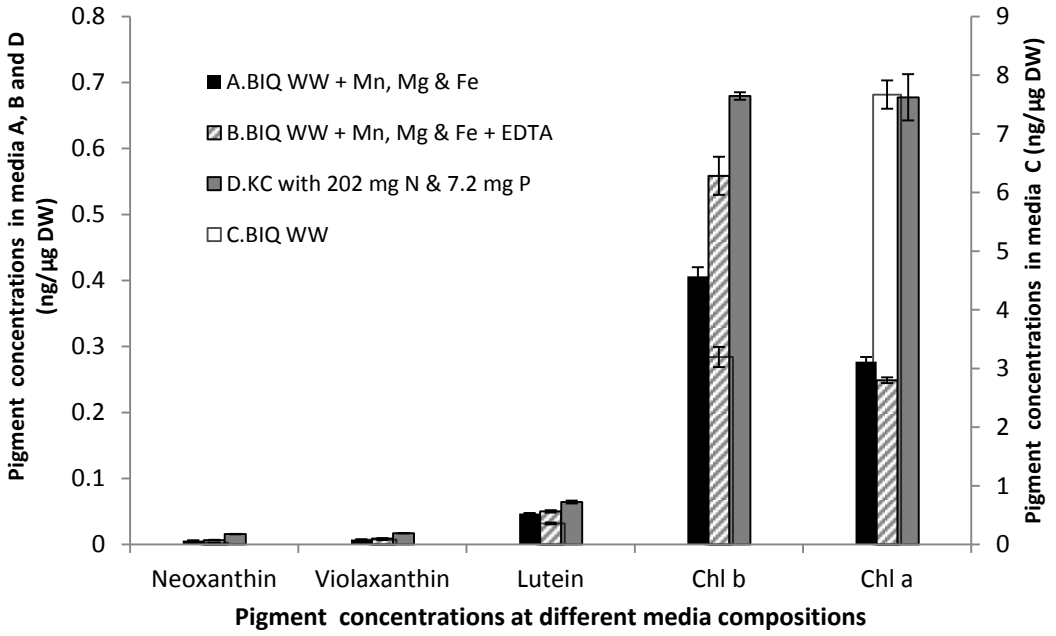


Figure 30. Pigment concentration of *A. obliquus* in BIQ wastewater media and in a control medium (KC). Mean values were calculated from triplicate ± standard deviation.

3.1.3 Repeated batch experiment of the BIQ wastewater

3.1.3.1 Characteristics of BIQ wastewater

Nutrient fluctuation emerged during the observation of 2 different wastewater sampling periods from BIQ (Table 29). Compared to the BIQ wastewater used in the previous experiment (Table 20), the Total Organic Carbon (TOC) concentration of the BIQ wastewater used in these repeated batch experiments was 2.01-2.52 times higher. However, the total nitrogen of the previous BIQ wastewater was 1.5-1.68 times higher than its concentration in the BIQ wastewater used in this repeated batch experiment. In addition, the total phosphorus

concentration of the previous BIQ wastewater (9.37 mg L^{-1}) was in the concentration range of the BIQ wastewater used in this repeated batch experiment (5.88-14.43 mg L^{-1}).

Table 29. Characteristics of each BIQ wastewater after fermentation, filtration, and UV sterilization at the BIQ

Parameter	The 2nd BIQ wastewater	The 3rd BIQ wastewater
TP (mgL^{-1})	5.88	14.43
TC (mgL^{-1})	533.75	
TOC (mgL^{-1})	345.88	275.37
TN (mgL^{-1})	127.43	115.20
NH_4-N (mgL^{-1})	115.8	89.37
NO_3-N (mgL^{-1})	1.13	
COD (mgL^{-1})		860.67

3.1.3.2 The first repeated batch experiment

Repeated batch experiments were designed to observe the ability of maintaining productivity of *A. obliquus* in several batch experiments. Autoclaved and non-autoclaved BIQ wastewater was compared to assess the ability of non-autoclaved BIQ wastewater to support also the growth of *A. obliquus*. During this first repeated batch experiment, 3 refill times of the 2nd BIQ wastewater were performed (Figure 31). It is clearly shown that the growth of *A. obliquus* in the BIQ wastewater (which was investigated by optical density and cell dry weight) tended to decrease from the first batch to the third batch (Figure 31).

Declined growth of *A. obliquus* in this repeated batch experiment is examined from productivity (Table 30) and specific growth rate (Table 31) values. Compared to the value of the first refill, productivity of *A. obliquus* in the sterilized BIQ wastewater (medium A) during the second and the third refill was only 62.21% and 30.6%, respectively. Likewise, the specific growth rate of *A. obliquus* in medium A in the second and the third refill was only 49.07% and 37.89%, respectively, compared to the first refill. Comparably, productivity of *A. obliquus* in the unsterilized BIQ wastewater (medium B) during the second and third refill was only 68.27% and 29.4%, respectively, while the specific growth rates were 53.96% and 32.37%, respectively, from the first refill.

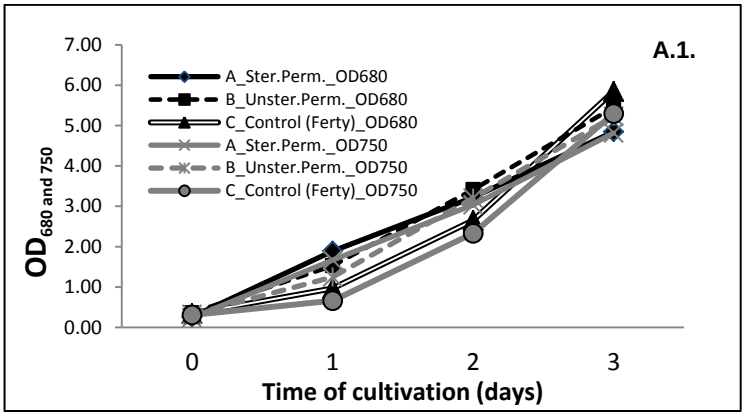
During the first refill, productivity of *A. obliquus* in sterilized BIQ wastewater was significantly 1.16 times higher, than in unsterilized wastewater (ANOVA $F=43.2$, d.f. 2,6, $p \leq 0.05$). Likewise, productivity in sterilized BIQ wastewater (medium A) was 1.057 and 1.21 times higher than in unsterilized BIQ wastewater (medium B), respectively for the second and the third refill. Similar to productivity, a higher growth rate of *A. obliquus* occurred in sterilized BIQ wastewater than in unsterilized BIQ wastewater during the first, the second and the third refill (1.16, 1.05 and 1.36 times, respectively) (Table 31). Nonetheless, statistically significant difference occurred only in the first refill (ANOVA $F=53.8$, d.f. 2,6, $p \leq 0.05$).

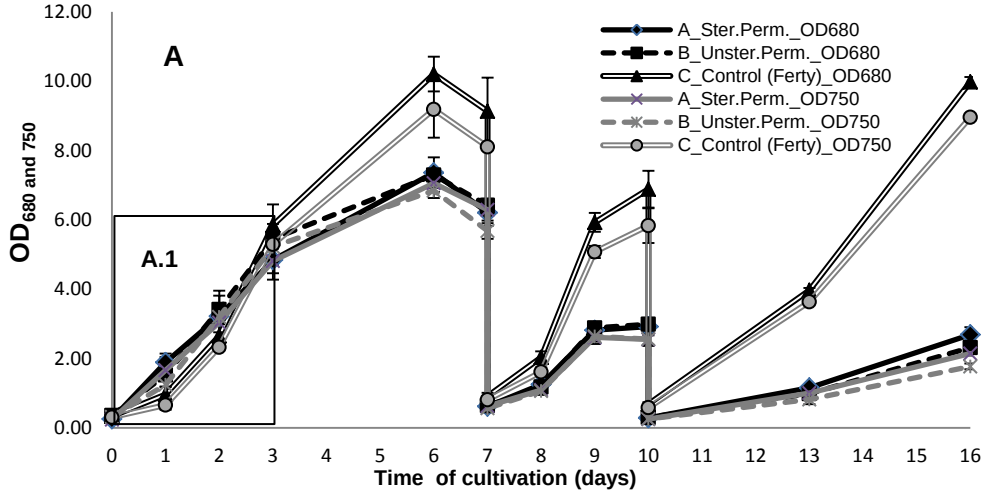
Table 30. Productivity of *A. obliquus* in 3 different refill media of BIQ wastewater and in a control (Ferty) during the first repeated batch experiment

	1st refill ($g\ L^{-1}\ d^{-1}$)	2nd refill ($g\ L^{-1}\ d^{-1}$)	3rd refill ($g\ L^{-1}\ d^{-1}$)
A. Sterilized Permeate	0.651±0.03	0.405±0.03	0.199±0.02
B. Unsterilized Permeate	0.561±0.02	0.383±0.02	0.165±0.02
C. Control (Ferty)	0.731±0.01	1.214±0.12	0.742±0.02

Table 31. Specific growth rate of *A. obliquus* in 3 different refill BIQ wastewater media and in control media (Ferty), based on CDW, during the first repeated batch experiment (d^{-1})

	1st refill	2nd refill	3rd refill
A. Sterilized Permeate	1.61±0.04	0.79±0.06	0.61±0.08
B. Unsterilized Permeate	1.39±0.04	0.75±0.04	0.45±0.09
C. Control (Ferty)	1.66±0.02	1.64±0.09	1.73±0.01





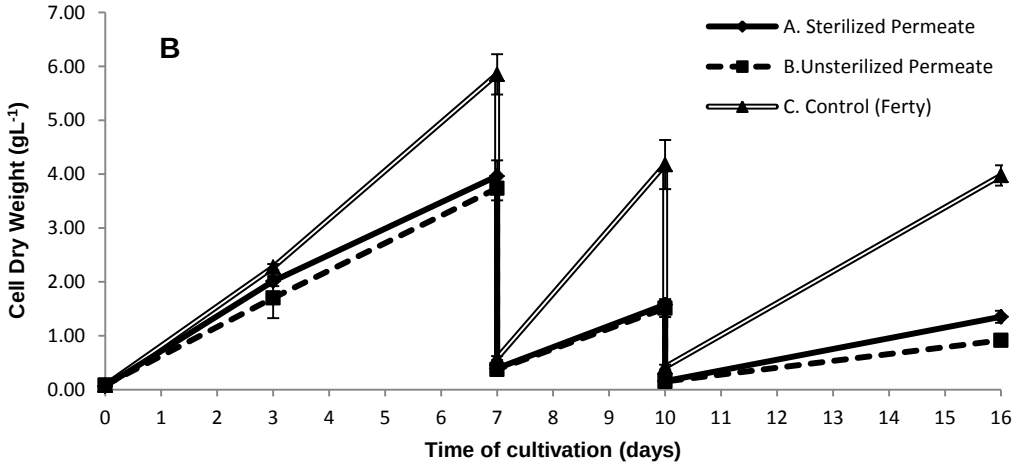


Figure 31. Growth of *A. obliquus* in sterilized and unsterilized BIQ wastewater and in a control medium (Ferty), determined by: A.Optical density; B.Cell Dry weight

Compared to the control, productivity and specific growth rates of *A. obliquus* in the BIQ wastewater decreased from the first to the third refill. The addition of elements during the second refill did not show any increase of growth. At the first refill, the highest productivity values of *A. obliquus* in sterilized BIQ wastewater was 89.06% compared to control, but then decreased to 33.36% and 26.82%, respectively at the second and third refill (Table 30). Likewise, the highest specific growth rates decreased from the first to the third refill from 96.99% to 48.17% and 35.26%, respectively (Table 31). Interestingly, for the first 2 days of experiment, higher optical density values of *A. obliquus* were observed in the BIQ wastewater than in the control (Figure 31-A.1.).

A lower photosynthetic activity of *A. obliquus* in the BIQ wastewater than in control medium was observed from the first to the third refill of the experiment (Figure 32). A reduction of the final Fv/Fm values of *A. obliquus* from the first to the third refill was observed in sterilized and unsterilized BIQ wastewater media. However, this decrease was also observed in the control medium.

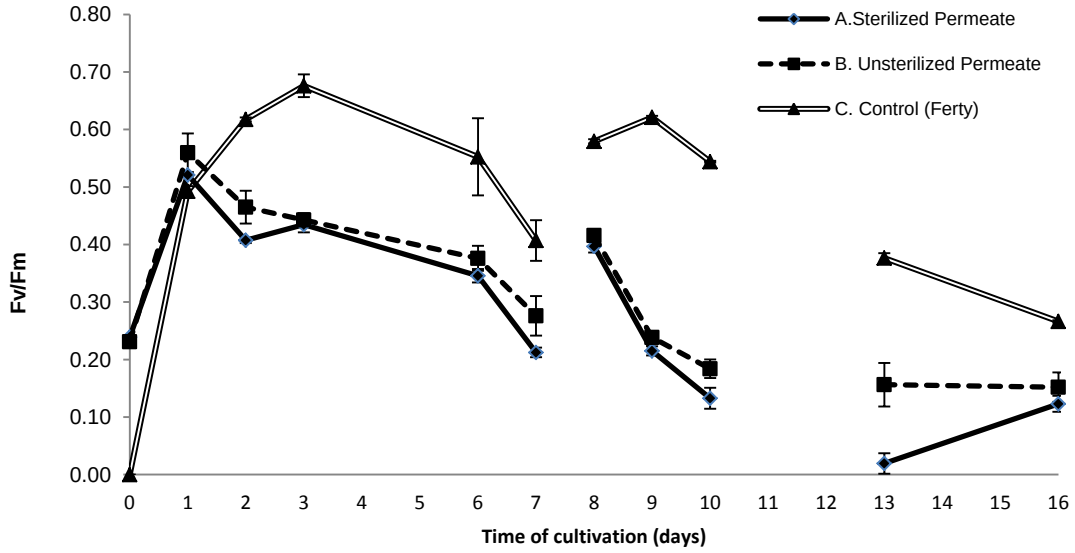


Figure 32. Photosynthetic activity of *A.obliquus* in sterilized and unsterilized BIQ wastewater and in a control medium (Ferty)

3.1.3.3 Recheck experiment of the result from the first repeated batch experiment

Based on results from the first repeated batch experiment, the repetition (recheck experiment) was carried out to observe the possible growth limitation by different micro elements. For this, all micro elements occurring in Ferty basis 1 medium were added. A phenomenon similar to that found in the previous experiment (3.1.3.2) was observed during the first 3 days of experiment, in which a higher optical density values (1.41, 1.55 and 1.3 times higher, respectively for day 1,2 and 3) occurred in BIQ wastewater (medium B) than in the control medium (Figure 33_A). However, from day-7, *A. obliquus* already reached the stationary phase in the BIQ wastewater, in contrast to the control. As also shown in the previous experiment, the end cell dry weight (CDW) of *A. obliquus* in the control medium was 1.26 times higher than that in the BIQ wastewater (Figure 33 B). Likewise, the optical density of *A. obliquus* at the end in the control medium was 1.32 times higher than in BIQ wastewater (Figure 33 A).

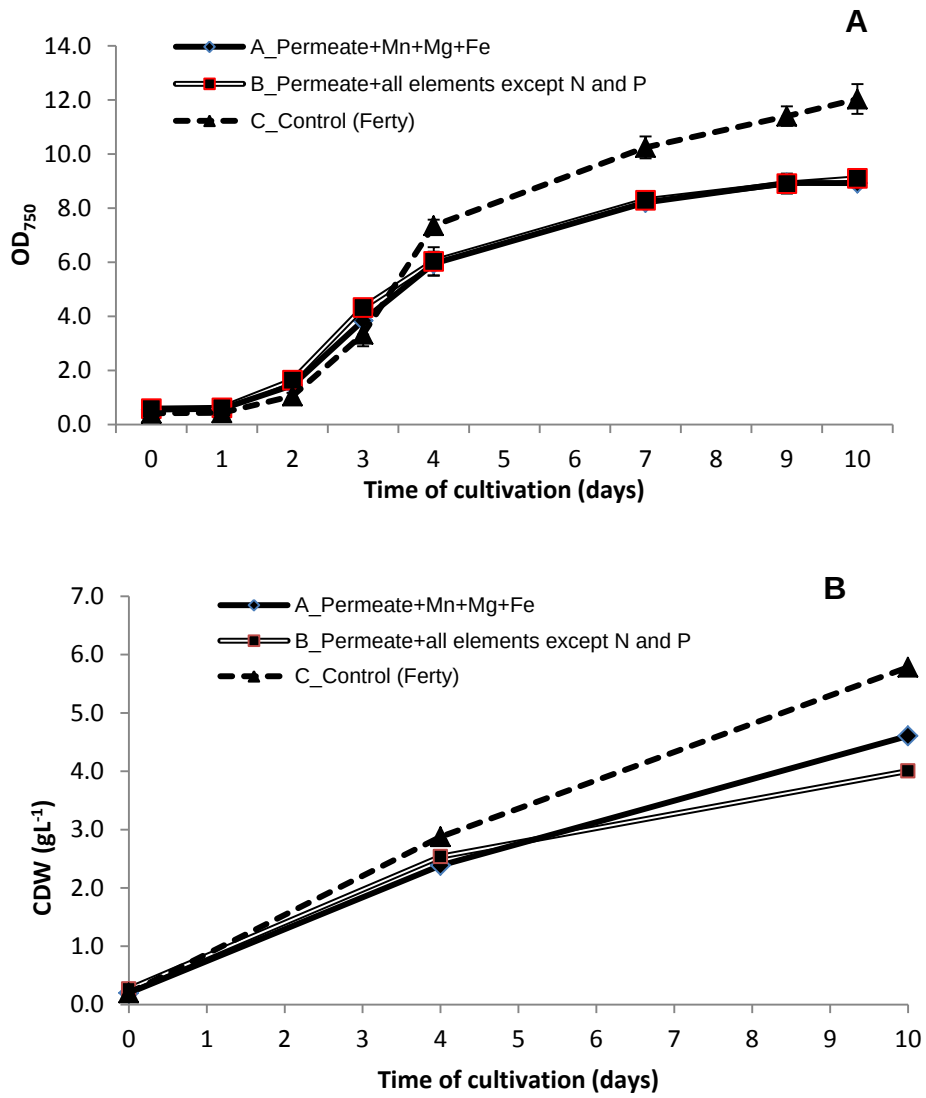


Figure 33. Growth of *A. obliquus* in different compositions of BIQ wastewater and in a control medium during a recheck experiment, observed by A: Optical Density and B: Cell Dry Weight

Compared to the control, the highest productivity and specific growth rate values of *A. obliquus* in BIQ wastewater were only 84.75% and 87.27%. No significant differences were observed in productivity (ANOVA F=20.584, d.f.2,6, p≥0.05) and specific growth rate (ANOVA F=39.57, d.f.2,6, p≥0.05) when additional elements were added to the BIQ wastewater (Table 32).

Table 32. Productivity and specific growth rate of *A. obliquus* in different compositions of BIQ wastewater and in a control medium in a recheck experiment

	Productivity ($g\ L^{-1}\ d^{-1}$)	Growth Rate in CDW (d^{-1})
A_Permeate+3 elements (Mn, Mg and Fe)	0.547±0.024	1.275±0.04
B_Permeate+all elements except N and P	0.567±0.032	1.265±0.025
C_Control (Ferty basis 1)	0.669±0.016	1.461±0.024

Photosynthetic activity of *A. obliquus* in the BIQ wastewater was slightly lower than those in the control medium (Figure 34).This finding is in accordance with lower productivity and growth rate of *A.obliquus* in BIQ wastewater than those in control, as described above.

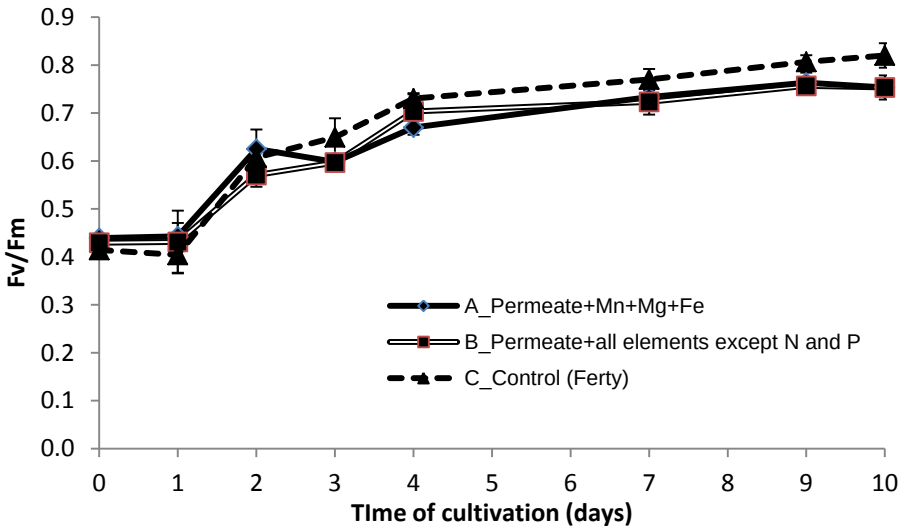


Figure 34. Photosynthethic activity of *A. obliquus* in different compositions of BIQ wastewater and in control medium in a recheck experiment

3.1.3.4 The second repeated batch experiment

The BIQ wastewater used in this experiment was collected during a different discharge period than the first repeated batch. Based on the results of the previous experiments, 3 possible limiting elements were also added to this experiment.

Growth of *A. obliquus* decreased again in the BIQ wastewater without additional elements (medium A) from the first to the third refill as shown in Figure 35. Although in the first refill, productivity and specific growth rate of *A. obliquus* in medium B were 1.016 and 1.021 times higher than those in BIQ wastewater without additional elements (medium A), no statistically significant differences were observed ((ANOVA F=49.98, d.f. 2,6, p≥0.05) and (ANOVA F= 76.078, d.f. 2,6, p≥0.05), for productivity and specific growth rate). However, lower productivity (Table 33) and specific growth rate (Table **34**) of *A. obliquus* was observed in medium A than medium B during the second and the third refill. In the second refill, additional elements significantly increased productivity and specific growth rate of *A. obliquus* 1.56 and 1.42 times higher, respectively, than without additional elements (ANOVA F=14.937, d.f. 2,6, p≤0.05 and ANOVA F=13.79, d.f. 2,6, p≤0.05, respectively for productivity and specific growth rate). Likewise, productivity and specific growth rate of *A. obliquus* in medium B were significantly 3.81 times higher (ANOVA F=259.318, d.f. 2,6, p≤0.05) and 4.22 (ANOVA F=226.328, d.f. 2,6, p≤0.05) than in medium A at the third refill. Unexpectedly, productivities and growth rates of *A. obliquus* in control medium (medium C) were much lower than in medium B, in which limiting

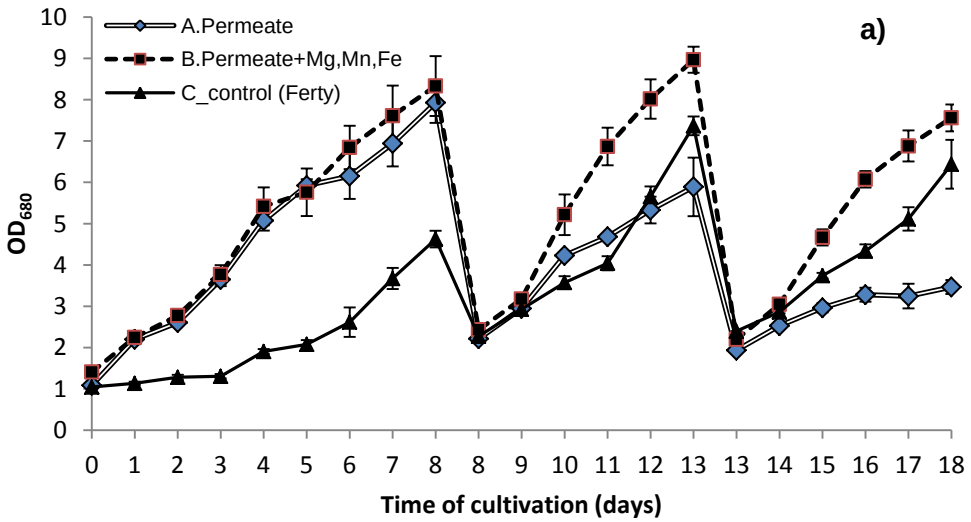
elements were added (Table 33 and Table 34). Productivity of *A. obliquus* in medium C was only 53.5%, 64.5% and 56.8% of that in medium B, while the specific growth rate of such alga in medium C was only 46.9%, 70.6% and 62.1% of those in medium B, respectively in the first, second, and third refill. At some data points, lower and fluctuating pH values were observed in medium C than in media A and B (Figure 36).

Table 33. Productivity of *A. obliquus* in BIQ wastewater with and without additional Mn,Mg and Fe and in a control medium during the second repeated batch experiment

	1st refill ($g\ L^{-1}\ d^{-1}$)	2nd refill ($g\ L^{-1}\ d^{-1}$)	3rd refill ($g\ L^{-1}\ d^{-1}$)
A_Permeate	0.254±0.022	0.268±0.051	0.105±0.009
B_Permeate +Mg,Mn,Fe	0.258±0.016	0.417±0.038	0.400±0.015
C_Control (Ferty)	0.138±0.010	0.269±0.020	0.227±0.022

Table 34. Specific growth rate of *A. obliquus* in BIQ wastewater with and without additional Mn, Mg and Fe and in a control medium), based on CDW, during the second repeated batch experiment

	1st refill (d^{-1})	2nd refill (d^{-1})	3rd refill (d^{-1})
A_Permeate	0.994±0.073	0.777±0.129	0.254±0.047
B_Permeate +Mg,Mn,Fe	1.015±0.049	1.106±0.064	1.071±0.027
C_Control (Ferty)	0.476±0.056	0.781±0.051	0.665±0.061



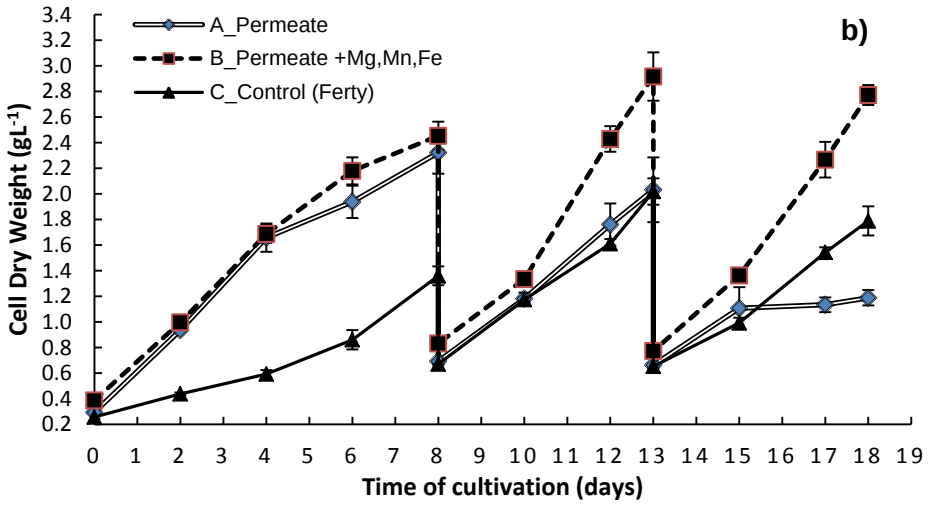


Figure 35. Growth of *A. obliquus* in BIQ wastewater with and without additional Mn, Mg, and Fe and in a control medium during the second repeated batch experiment: a) Optical Density ; b) Cell Dry Weight

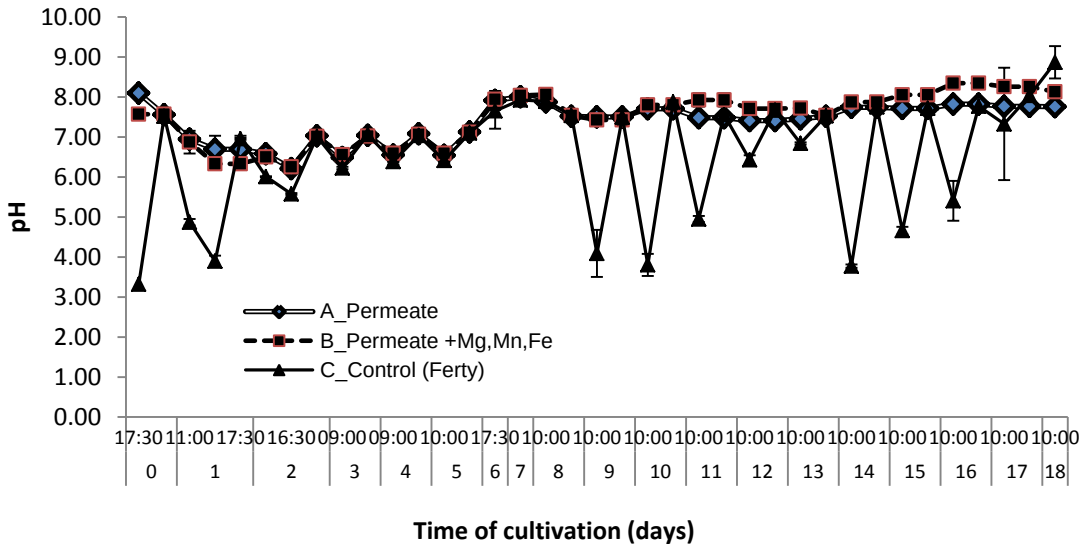


Figure 36. pH fluctuation in the different media compositions of BIQ wastewater and a control medium

Compared to the first refill, a decreased photosynthetic activity of *A. obliquus* was observed in medium A at the second and the third refill (Figure 37). At the end of the second and the third refill Fv/Fm values of *A. obliquus* in the BIQ wastewater without additional elements were only 68**%** and 46% of those in the BIQ wastewater with additional elements (Table 35).

Table 35. Photosynthetic activity (Fv/Fm) of *A. obliquus* at the end of the first, second, and the third refill of the second repeated batch experiment

	1st refill	2nd refill	3rd refill
A_Permeate	0.544±0.017	0.440±0.012	0.305±0.074
B_Permeate +Mg,Mn,Fe	0.595±0.007	0.647±0.007	0.667±0.003
C_Control (Ferty)	0.553±0.066	0.623±0.007	0.629±0.013

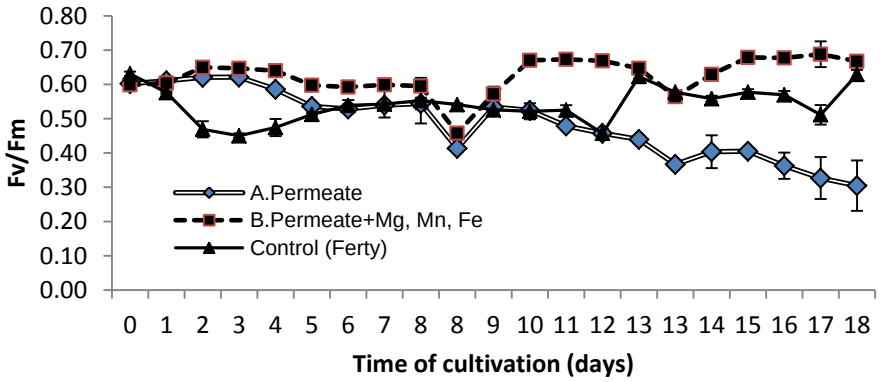


Figure 37. Photosynthetic activity of *A. obliquus* in BIQ wastewater with and without additional elements and in a control medium during the second repeated batch experiment

During the first and the second refill period, more than 90% of total nitrogen (TN) and total phosphorous (TP) was removed from the BIQ wastewater (Table 36). Nonetheless, only about half of the TN was removed from the BIQ wastewater without additional elements during the third refill (Table 36).

Table 36. Total Nitrogen and Total Phosphorous Removal (%) in the BIQ wastewater with and without additional elements in a control medium during the 2nd repeated batch experiment

	1st refill		2nd refill		3rd refill	
	TN	TP	TN	TP	TN	TP
A_Permeate	92.26±0.66	97.02±0.56	95.10±0.30	90.7±3.49	54.39±0.76	98.55±0.92
B_Permeate +Mg,Mn,Fe	94.17±0.38	98.38±0.28	95.66±0.10	96.5±0.30	95.25±0.24	99.62±0.07
C_Control (Ferty)	93.24±4.23	97.74±0.31	97.81±0.03	96.07±0.31	95.54±1.71	99.59±0.02

Total nitrogen removal in medium B (Figure 38-a) was in accordance with biomass production of *A. obliquus* in such media, in which a relative stable growth rate (Table 34) and productivity (Table 33) were observed. Likewise, such removal was in accordance with growth of *A. obliquus* in medium A at the third refill, where the lowest productivity and growth rate went with the lowest percentage of TN removal.

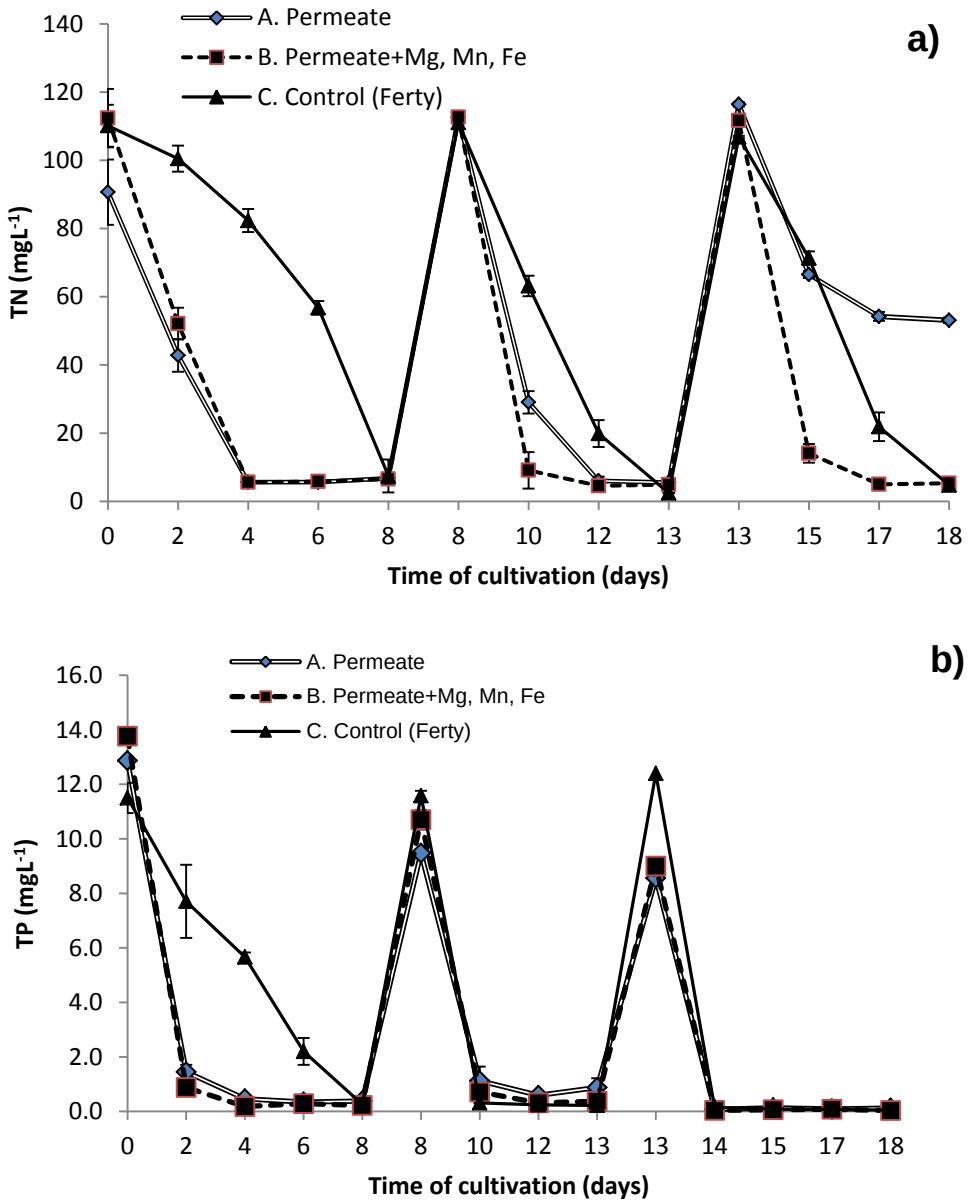


Figure 38.Total nitrogen (TN) (a.) and Total phosphorus (TP) (b.) concentration in the media of BIQ wastewater with and without additional elements

Although at the last refill, productivity and specific growth rate of *A. obliquus* in medium A was only 41.33**%** and 25.55 % of that of the first refill, total phosphorus (TP) was removed efficiently. Only 0.124 mg L^{-1} remained at the end of the third refill (Figure 38-b). More than 96% TP was removed in the BIQ wastewater with additional elements from the first refill until the third refill (Table 36).

In general, COD concentration during the experiment followed the DOC pattern. COD decreased by 80% in BIQ wastewater with and without additional elements (Figure 39-b) as well as more than 60% Dissolved Organic Carbon (DOC) (Figure 39-a) was removed at the first,

second and third refill. Less than 50 mg L^{-1} of DOC concentration was observed in the control medium. The COD concentration in medium C was not measured as there was no organic C from wastewater in the control.

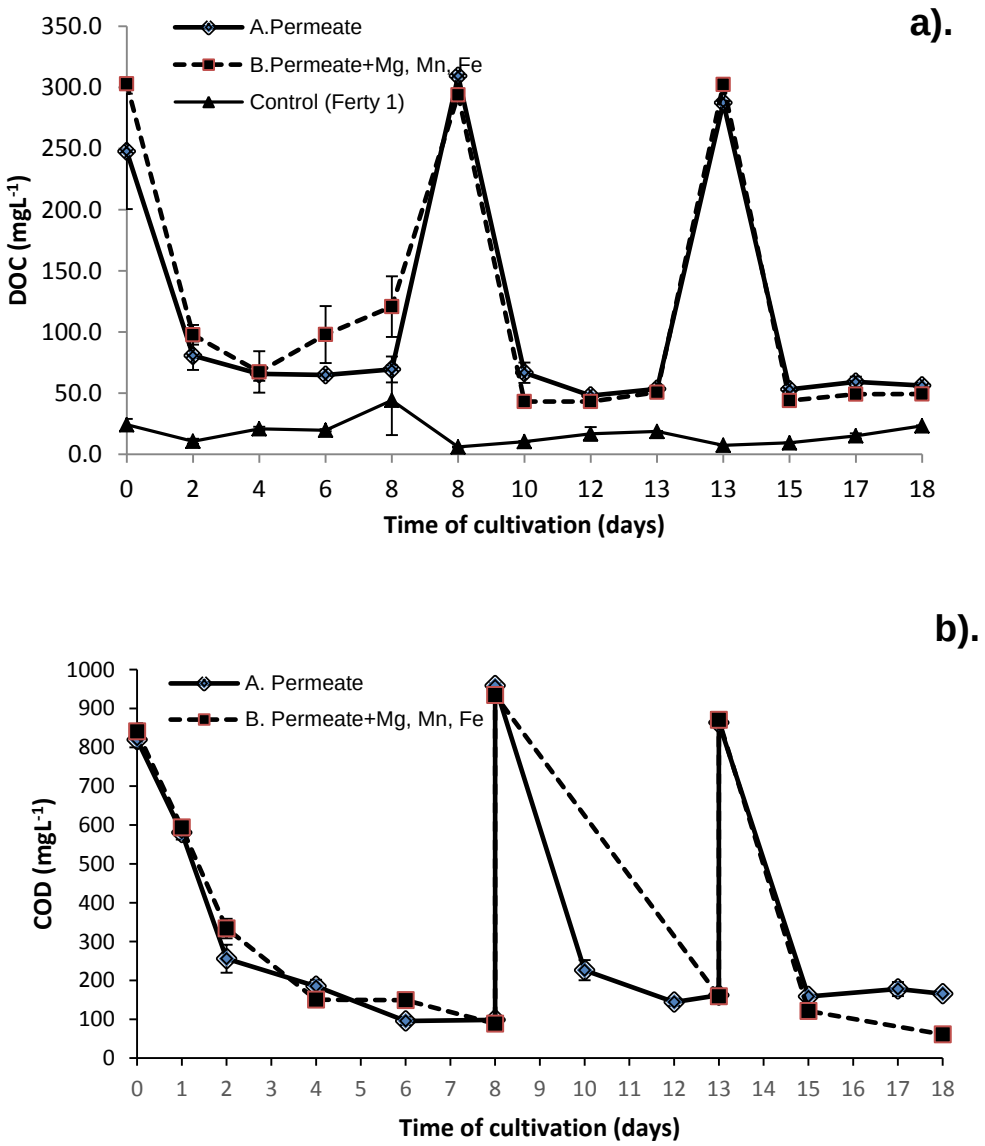
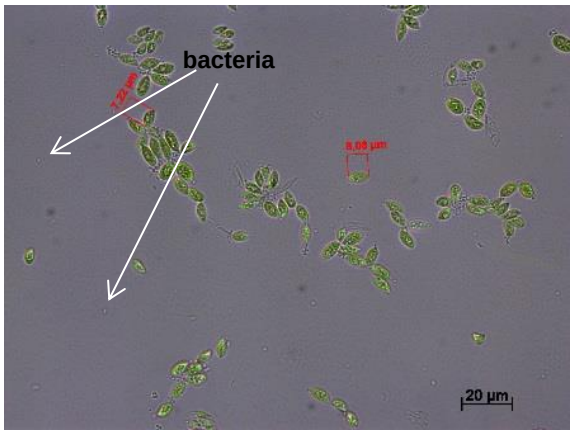


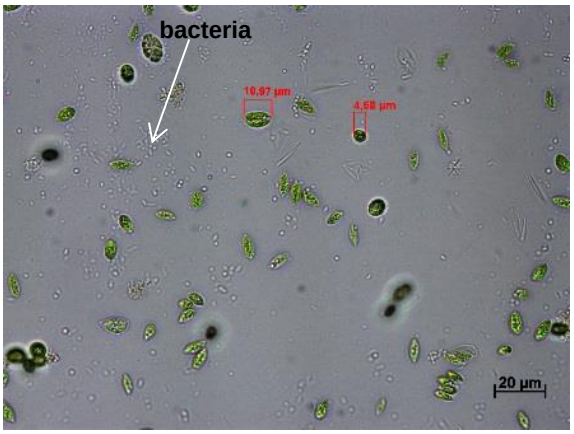
Figure 39. Dissolved Organic Carbon (a.) and Chemical Oxygen Demand (b.) in the BIQ wastewater during the second repeated batch experiment

Bacterial contamination was observed in media A and B, while nearly no contamination was visible in medium C, which was previously sterilized at 121^{0}C (Figure 40).

a).



b).



c).

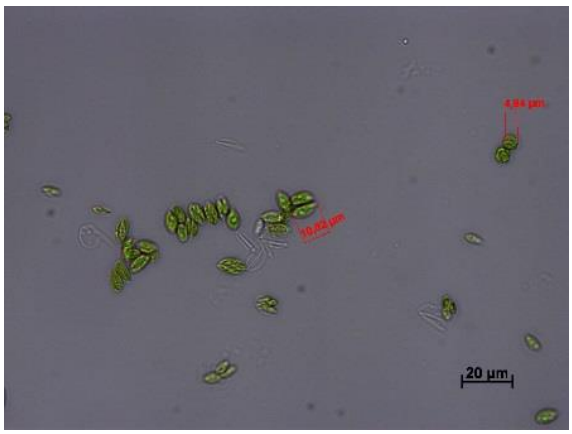


Figure 40 Light microscope picture of *A. obliquus* cultivated in: a). BIQ wastewater; b).BIQ wastewater with additional elements; c). Ferty media

At the end of the experiment, COD, TN and TP concentrations from BIQ wastewater with additional elements (medium B) fulfilled the requirements set by European Council Directive 91/271/EEC of 21 May 1991, concerning the urban waste-water treatment (EEC Council 1991) (Table 37).

Table 37. Comparison of wastewater quality after algal cultivation in BIQ wastewater and some parameters determined in the regulation for discharging wastewater

	COD (mgL^{-1})	TN (mgL^{-1})	TP (mgL^{-1})
Requirements for discharges from urban wastewater treatment plant	125	10	1
A. BIQ wastewater	166	53	0.124
B. BIQ wastewater+elements	61	5.3	0.034

3.1.4. Trophic mode experiment

3.1.4.1. Wastewater characteristics

Compared to BIQ wastewater used in the previous experiments, TOC concentrations in the BIQ wastewater used in this experiment was 3.35-8.46 times higher (Table 38). On the contrary, TN was 24.9**%**-41.8% of the concentration of the previous experiments while TP concentration was 1.2-3 times higher.

Table 38. Characteristics of the 4th BIQ wastewater after fermentation, filtration and UV sterilization at the BIQ

Parameter	Concentration (mg/L)
TOC	1158.60
TC	1242.42
TN	48.12
TP	17.52
$N\text{-}NH_4$	44.80
NH4	57.60
$N\text{-}NO_3$	1.12
NO3	4.94

3.1.4.2. Growth

Although the exponential phase of *A. obliquus* in trophic mode B (BIQ wastewater+N_2+light) (Table 6) just began on day-5 (Figure 41), productivity in this trophic mode was 1.3 times higher than in trophic mode C (Table 39). These results support my hypothesis that organic carbon in the BIQ wastewater is able to support the growth of *A. obliquus* and substitute the inorganic carbon as a carbon source. Nevertheless, light played an important role in the growth of *A. obliquus*. However, the highest productivity was achieved when *A. obliquus* grew mixotrophically (in trophic mode A) and almost no growth was observed in the heterotrophic mode (trophic mode D).

Table 39. Productivities of *A. obliquus* in different trophic modes

	A. BIQ WW+CO_2+light	B. BIQ WW+N_2+light	C. Modif.KC+light+N_2	D. BIQ WW+synthethic air
Productivity ($g\ L^{-1}\ d^{-1}$)	0.112±0.015	0.062±0.015	0.049±0.012	0.018±0.008
The highest Cell Dry Weight ($g\ L^{-1}$)	1.285±0.070	0.466±0.066	0.669±0.061	0.217±0.040

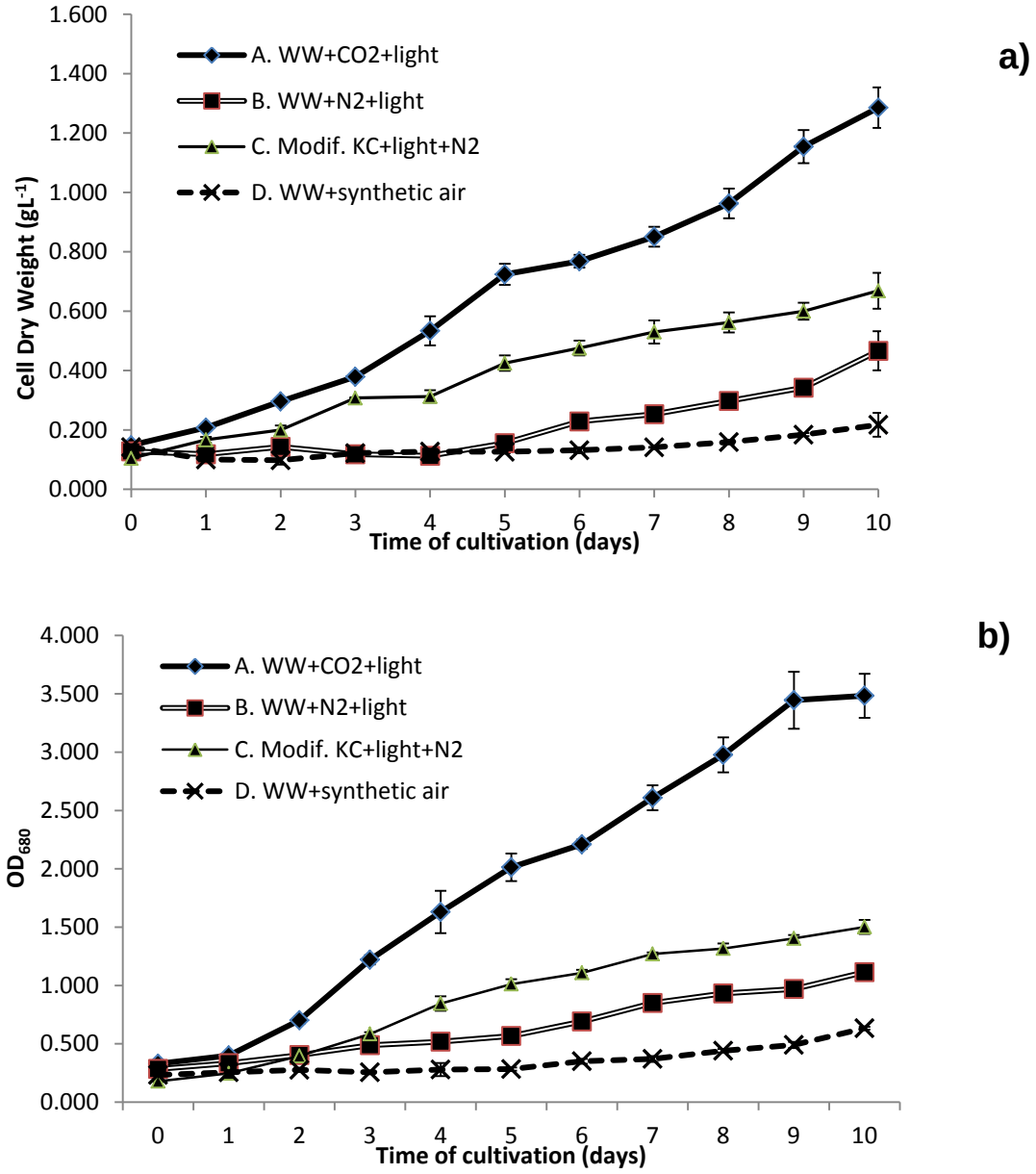


Figure 41. Growth of *A. obliquus* in different trophic modes, based on: a) Cell dry weight; b) Optical Density

The exponential phase started at day-5 in trophic mode B is indicated by photosynthesis activity that begins increasing on the same day (Fv/Fm values was 0.436-0.467 at day-1 until day-4 and started increasing from 0.498 at day-5 until 0.61 at the end of experiment without any impairment) (Figure 42). As also shown by the growth pattern, the highest Fv/Fm value in general throughout the experiment was observed in trophic mode A. A similar pattern of photosynthetic activity was also observed in trophic mode C, but only until day-7, then it reached relatively stable values. In trophic mode D, relatively stable low values of Fv/Fm were shown by *A. obliquus*.

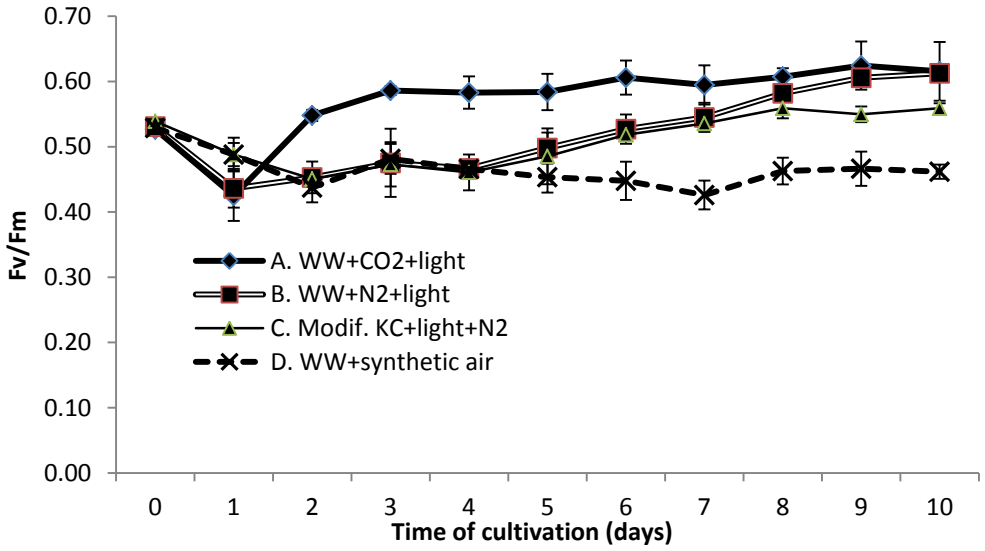


Figure 42. Photosynthetic activity of *A. obliquus* in different trophic modes

After 24 hours of cultivation, the oxygen evolution rate of *A. obliquus* in trophic mode A increased relatively until day-4 (91.903 µmol L^{-1} gDW^{-1} $min.^{-1}$), but decreased to 56.5% at day-5 and declined to 32.76% at the end of experiment. The increase of the oxygen evolution rate was followed by an increase of respiration rate after 24 hours of cultivation until day-4 (14.29 µmol L^{-1} gDW^{-1} $min.^{-1}$). Furthermore, respiration rates decreased to 4.49 µmol L^{-1} gDW^{-1} $min.^{-1}$ at the end of the experiment (Figure 43).

After 24 hours of cultivation, oxygen evolution rates of *A. obliquus* of trophic mode B and C fluctuated until day-3 with the highest rates at day-2 of 60.12 and 64.354 µmol L^{-1} gDW^{-1} $min.^{-1}$, respectively, in trophic mode B and C. These values are only 65.24**%** and 69.84% of the highest oxygen evolution rate in trophic mode A. Respiration rates of *A. obliquus*, both in trophic mode B and C fluctuated until day-2 but started increasing at day-3 and achieved their maximal values of 24.38 and 23.76 µmol L^{-1} gDW^{-1} $min.^{-1}$at day-4 (for trophic mode C) and day-5 (for trophic mode B), respectively. These respiration rates were 1.7 and 1.66 times higher (respectively in trophic mode C and B) than in trophic mode A (Figure 43). After day-5, the respiration rate of *A. obliquus* in trophic mode B and C fluctuated slightly, but remained higher than in trophic mode A by factors of between 1.84-4.54 and 2.62- 3.19, respectively for trophic mode B and C.

The highest range of oxygen evolution rates (17.6-42.81 µmol L^{-1} gDW^{-1} $min.^{-1}$) of *A. obliquus* in trophic mode D was observed until day-3 of cultivation. Afterward, oxygen evolution rates declined to 2.002 µmol L^{-1} gDW^{-1} $min.^{-1}$ at day-4 and only 1.18-2.8 µmol L^{-1} gDW^{-1} $min.^{-1}$ of oxygen evolution rates was observed until the end of the experiment. Before day-4 of cultivation, relatively high fluctuation of respiration rates (2.99-6.65 µmol$L^{-1}$$gDW^{-1}$$min.^{-1}$) was

observed by *A.obliquus* in trophic mode D. Afterward, slight and low fluctuation of respiration rates between 1.973-4.01 μmol L^{-1} gDW^{-1} $min.^{-1}$ were determined (Figure 43).

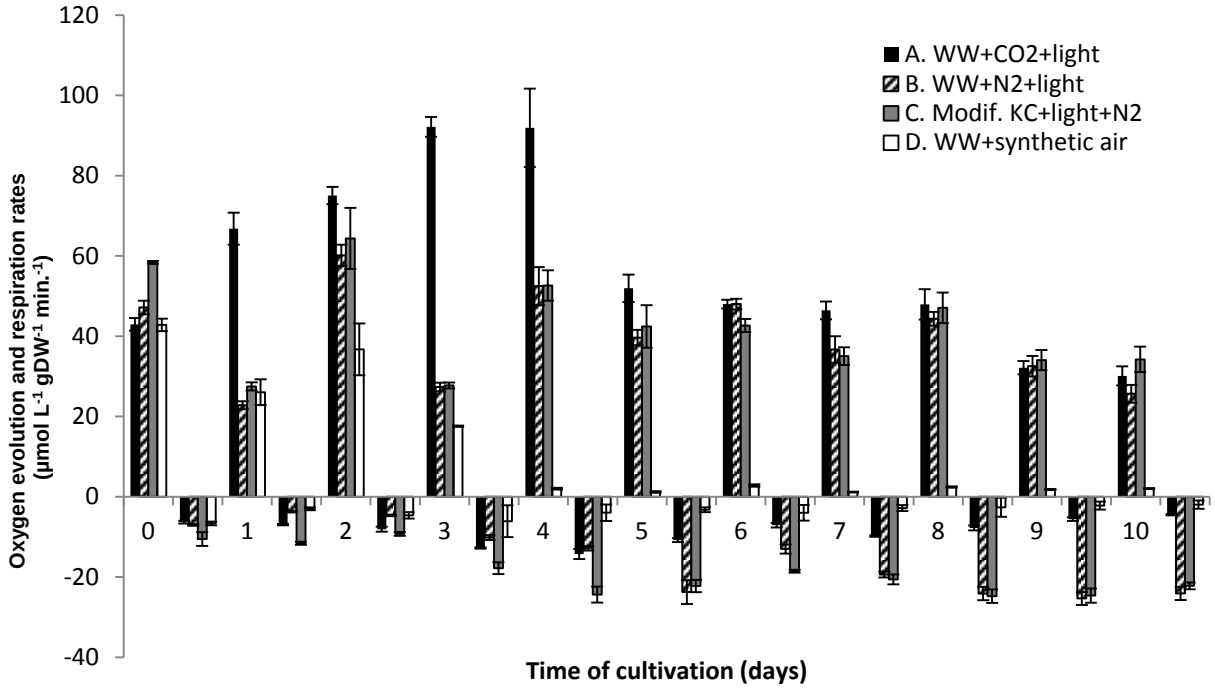


Figure 43. Rates of oxygen evolution and respiration of *A. obliquus* in different trophic modes

3.2. Outdoor scale

3.2.1. High-light outdoor experiment

During this experiment, the highest light intensity which occurred at the southwest photobioreactor façade was 1156.43 μmol photons m^{-2} s^{-1}, while on the southeast photobioreactor façade it reached 1535.05 μmol photons m^{-2} s^{-1} (Figure 44). This irradiance was 2.75-3.65 times higher than that used during the previous experiments in the laboratory scales.

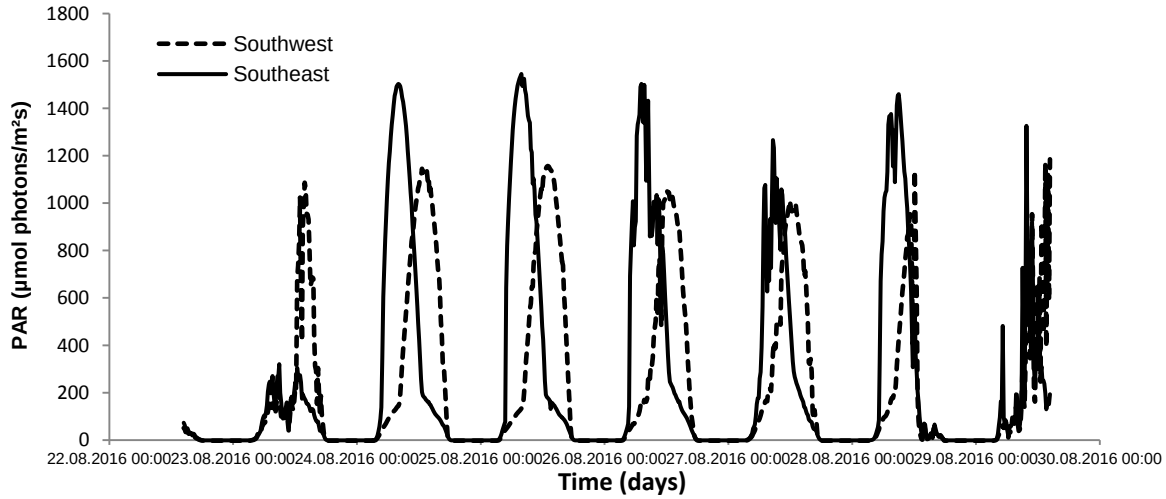


Figure 44. Light Intensity at the BIQ façades

The exponential phase of *A. obliquus* in BIQ wastewater started after 24 hours and reached its stationary phase at day-3 of the experiment (Figure 45). During this short experimental period, no elemental limitation was observed. The productivity and specific growth rate of *A. obliquus* in the BIQ wastewater without additional elements (L4) was 1.12 and 1.2 times higher, respectively, than in BIQ wastewater with additional elements (L3) (Table **40**).

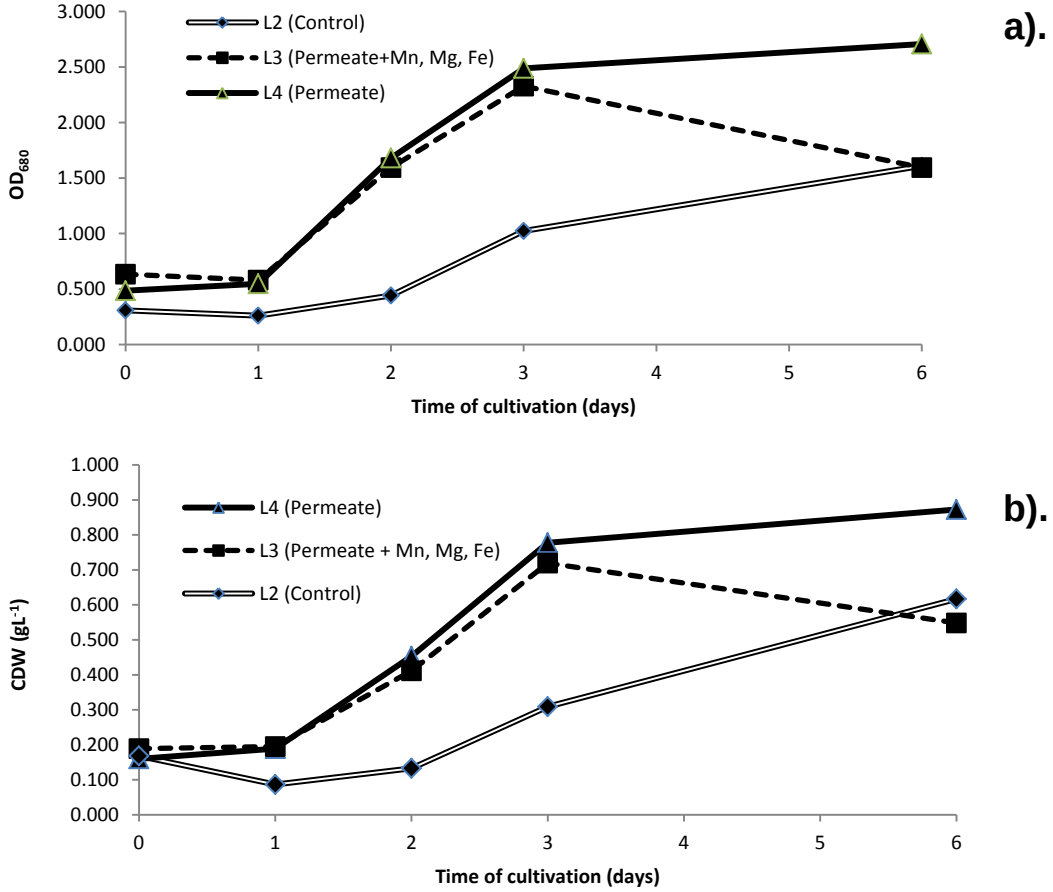
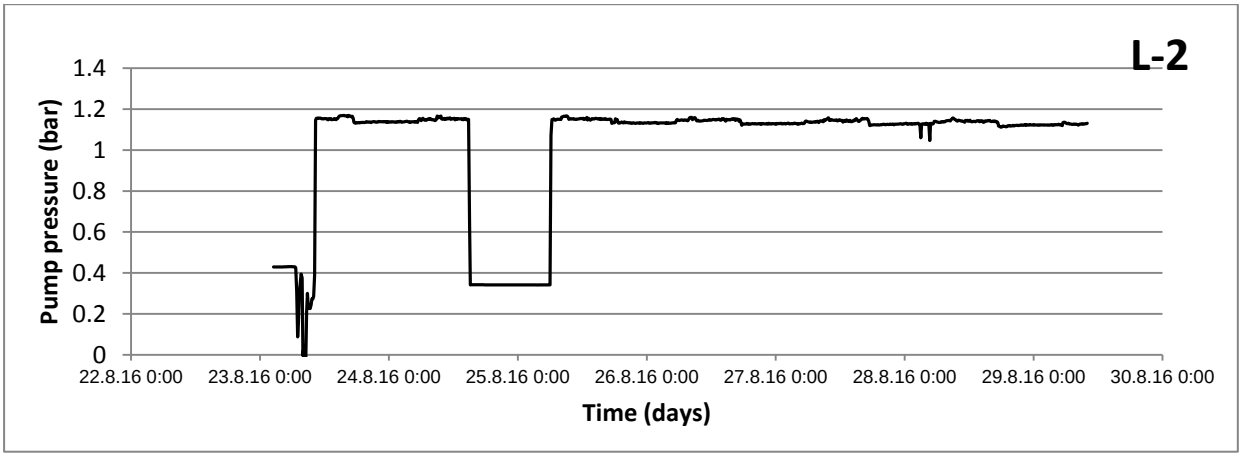
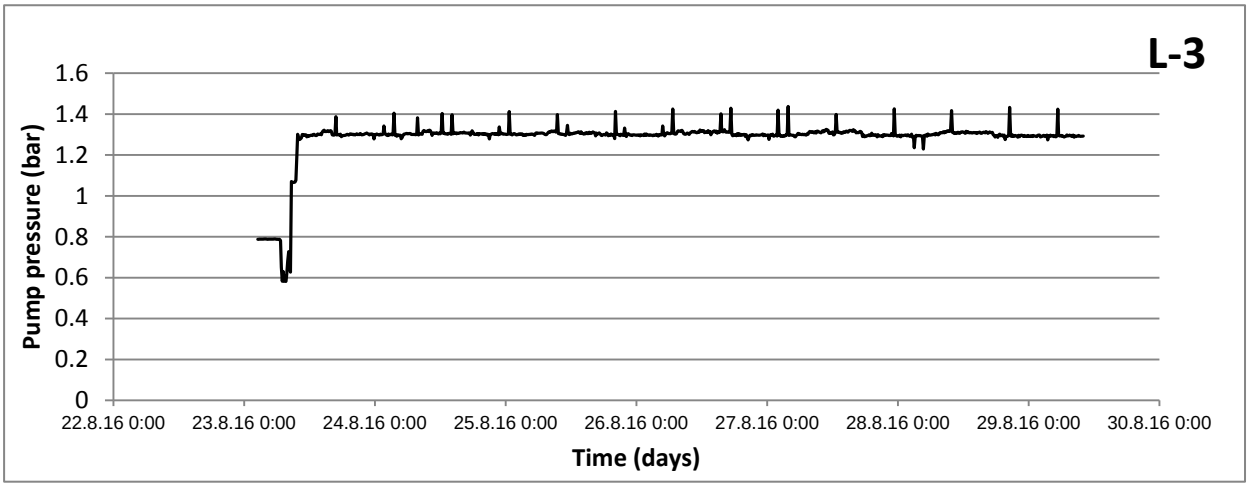


Figure 45. Growth of *A. obliquus* in the first experiment at the photobioreactor façade BIQ Algenhaus: a).Optical Density; b). Cell dry weight

Low productivity of *A. obliquus* in control medium (L2) (Ferty Basis 1, Planta Dünge Mittel GmBH, Germany) may have been caused by a decrease in pump pressure during the second day of the experiment (Figure 46). At day-6 of experiment, the circulation system in line-3 of the southeast façade did not work. Hence, biomass production decreased about 24% compared to day-3 (Figure 45).





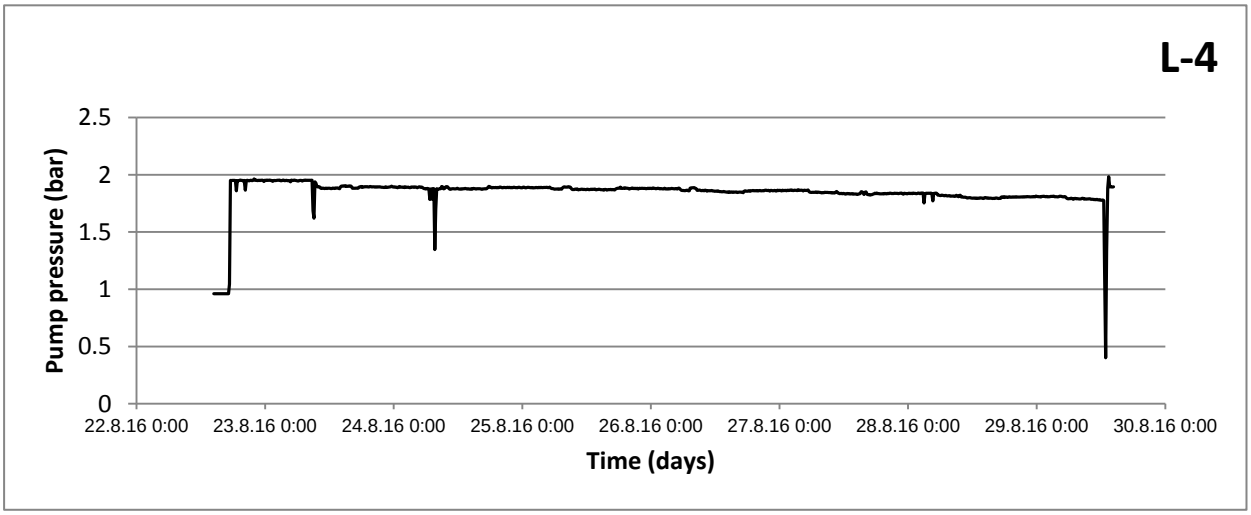


Figure 46. Pump pressure of each line in the BIQ photobioreactors

Table 40. Productivity and specific growth rate of *A. obliquus* in photobioreactor façades during the high-light experiment at BIQ Algenhaus, based on CDW.

	Productivity ($g\ L^{-1}\ d^{-1}$)	μ (d^{-1})	Area Productivity ($gm^{-2}day^{-1}$)
L4 (Permeate)	0.29	0.581	5.88
L3 (Permeate + Mn, Mg, Fe)	0.26	0.488	5.24
L2 (Control)	0.11	0.047	2.22

Figure 47 shows the time course of the photosynthetic activity of *A. obliquus* in all lines during the experiment. Fv/Fm increased and reached the maximum at day-2 (0.636 and 0.659, respectively for Line 3 and 4) and day-3 (0.632 for Line 2). The decrease of Fv/Fm after day-3 may be attributed to the onset of the stationary phase that can be figured out from L4 (Figure 45).

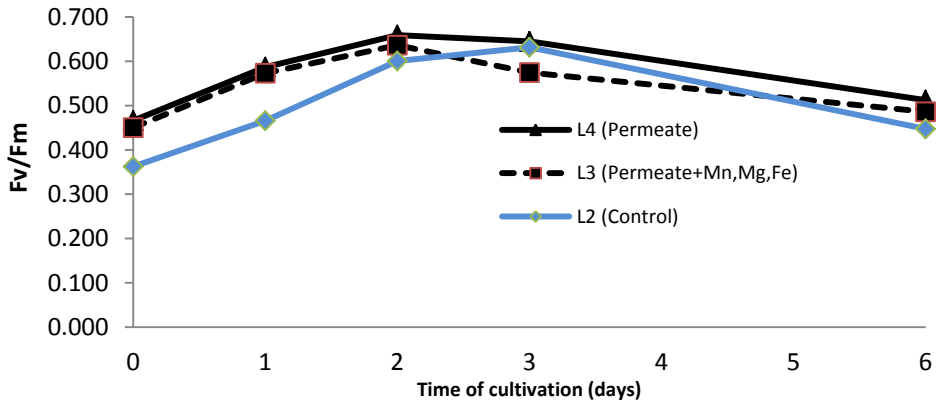


Figure 47. Photosynthetic activity of *A. obliquus* in the BIQ photobioreactors

During the experiment, total nitrogen concentration decreased below 5.5 mg L^{-1} and faster in line 3 and 4 than in line 2 (Figure 48). The lowest TN removal rate was in line 4 in which no limited elements were added (Table 41). Similarly, the lowest TP removal rate was observed in line 4 (Table 41) and remained at 57.86% of the initial TP concentration (Figure 48).

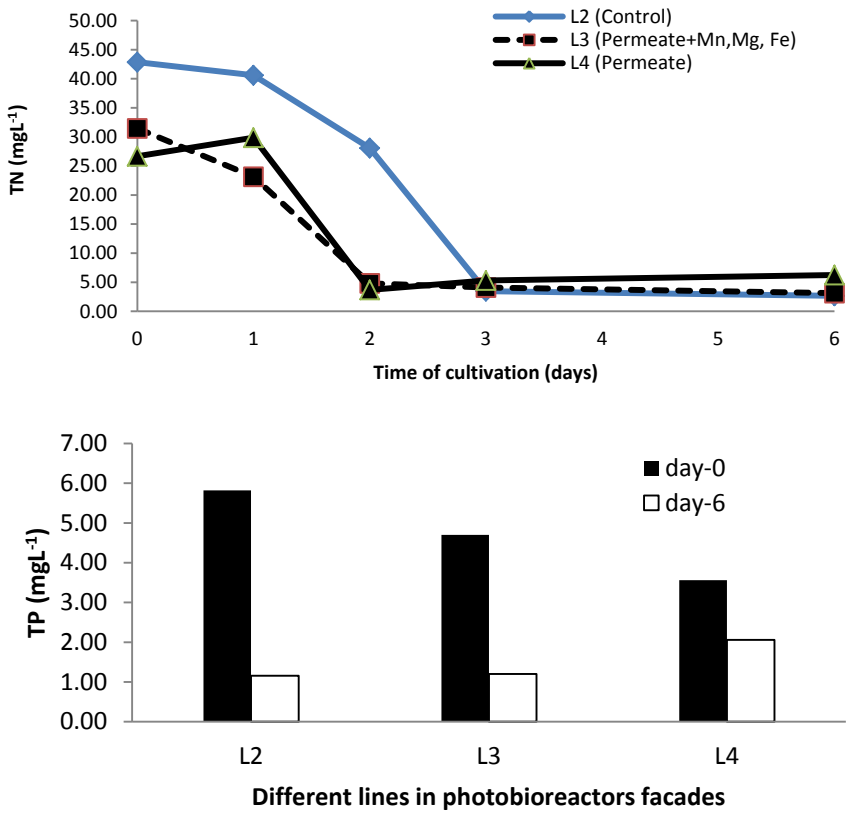


Figure 48. Total Nitrogen and Total Phosphorus concentration in the photobioreactor façades at BIQ

Table 41. Nitrogen and Phosphorus removal rate from the BIQ wastewater after cultivation with *A. obliquus*

	$TN_{(mgL^{-1}d^{-1})}$	$TP_{(mgL^{-1}d^{-1})}$
L2 (Control)	13.13	0.78
L3 (Permeate+Mn,Mg, Fe)	13.33	0.58
L4 (Permeate)	11.50	0.25

During the experiment, the pattern of Dissolved Organic Carbon (DOC) concentration in line 2 and line 4 were similar to the pattern of Chemical Oxygen Demand (COD) in these lines (Figure 49). A similar pattern between DOC and COD was also observed in line 3, although a slight increase of DOC concentration was visible at day-2 (1.14 times higher than day-1).

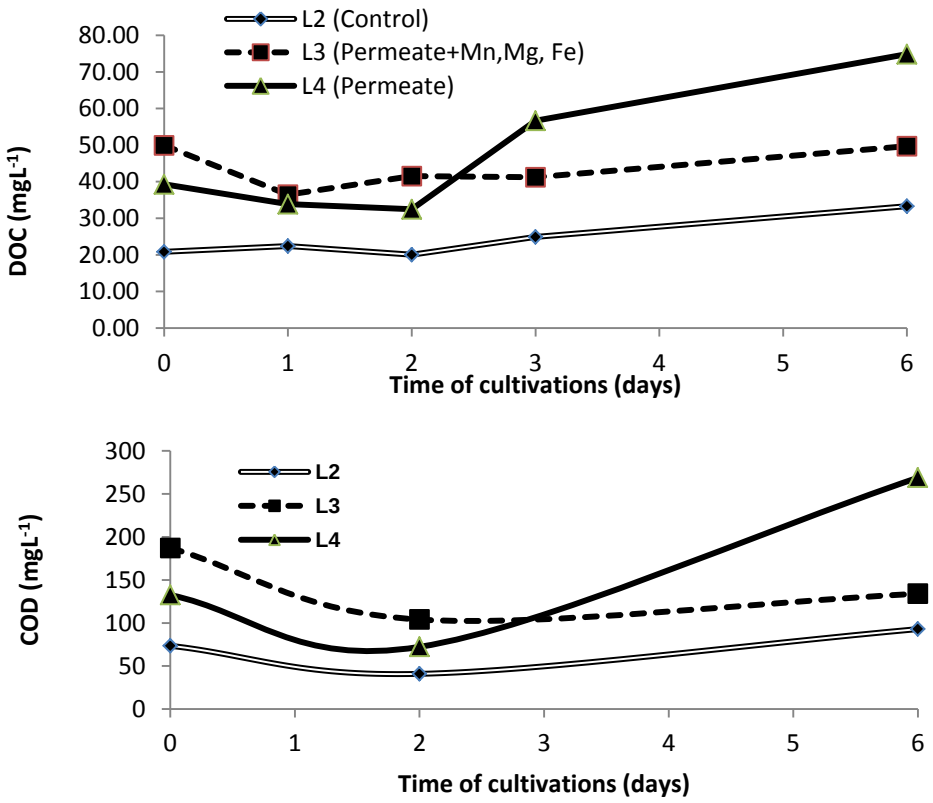


Figure 49. Dissolved Organic Carbon and Chemically Oxygen Demand in the photobioreactor façade at BIQ

3.2.2. Low-light outdoor experiment

Unlike to the high-light experiment, where irradiances reached more than 1000 µmol photons m^{-2} s^{-1}, such peaks occurred only in 2 of 13 experiment days (Figure 50). Generally, irradiances were below 400 µmol photons m^{-2} s^{-1} and even in 5 of those days they were below 200 µmol photons m^{-2} s^{-1}.

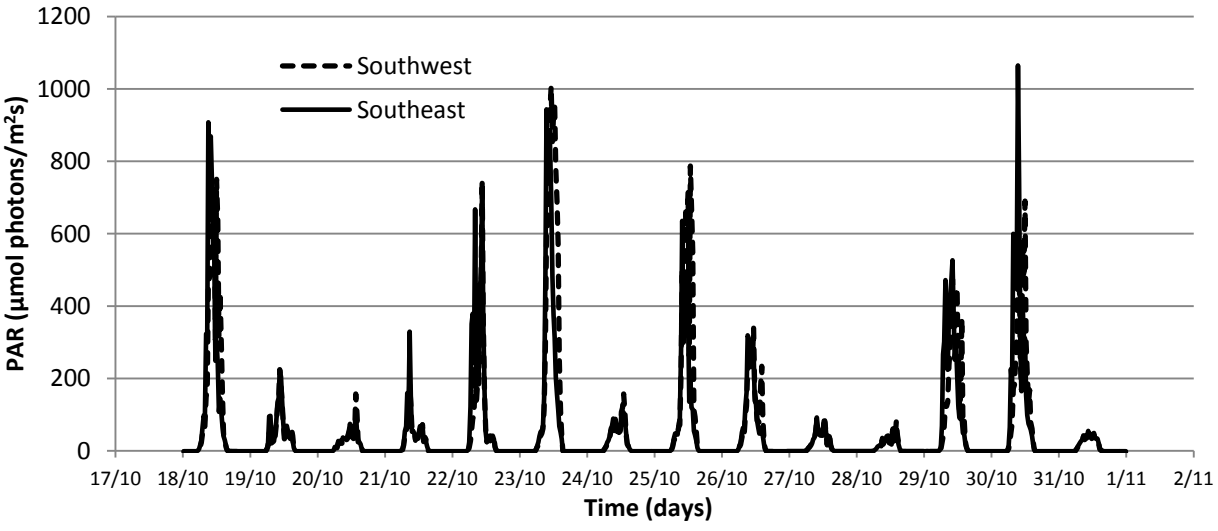


Figure 50. Photosynthetic Active Radiation in BIQ photobioreactors during the low-light experiment

In this experiment, a flushing procedure was carried out to resuspend settled cells back within circulation. Without flushing, no growth was observed until day-6 of the experiment (Figure 51). Growth of *A. obliquus* began after the flushing mechanism worked from day-7.

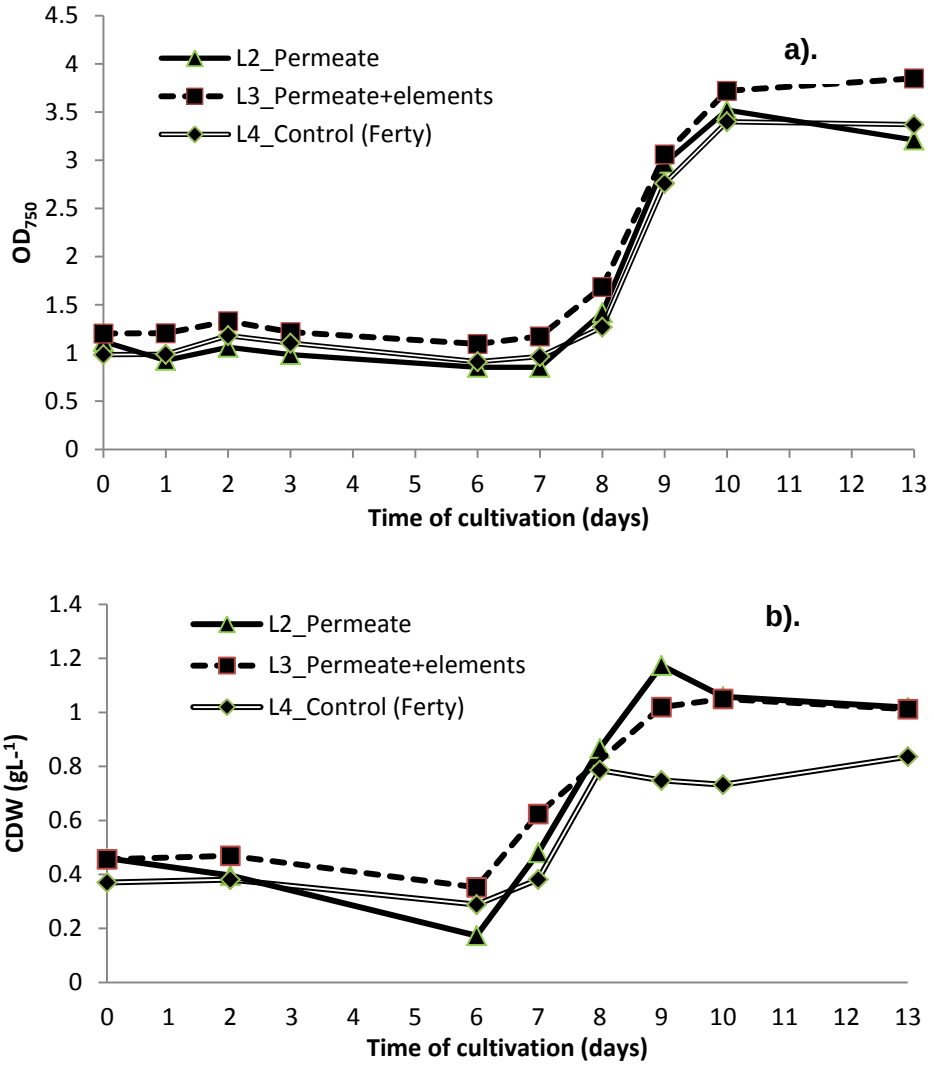


Figure 51. Growth of *A. obliquus* in the second experiment at BIQ Algenhaus photobioreactor façades: a).Optical Density; b). Cell dry weight

Similar to the experiment with high-light intensities, no element limitation was observed in the BIQ waste water used in this experiment. Three days after the initiation of the flushing system, stationary phase commenced in all lines (Figure 51). Without additional elements, productivity and specific growth rate were 1.5 and 2.02 times higher, respectively, than in wastewater with additional elements (Table 42). However, productivity and growth rates (of line 2 and line 3) were 1.46-2.18 and 2.94-5.96 times higher than in the control.

Table 42. Productivity and specific growth rate of *A. obliquus* in photobioreactor façades during the low light intensity experiment at the BIQ Algenhaus, based on CDW.

	Productivity ($g\ L^{-1}\ d^{-1}$)	Growth rate (d^{-1})	Area Productivity ($g\ m^{-2}\ day^{-1}$)
L2_Permeate	0.334	0.745	6.67
L3_Permeate+elements	0.223	0.368	4.45
L4_Control (Ferty)	0.153	0.125	3.07

Compared to the high-light experiment (Table **40**), the maximal productivity and growth rates of *A. obliquus* were 1.15 and 1.28 times higher. Likewise, productivity and specific growth rate in the control medium of this low-light experiment was 1.39 and 2.66 times higher, respectively, than that in high-light experiment.

At the beginning of this experiment, a low Fv/Fm value (0.295) of *A. obliquus* was observed in line-2 without addition of elements, but this increased to 0.589 in 24 hours. After 24 hours, the Fv/Fm values of *A. obliquus* in the BIQ wastewater media (with and without additional elements) were similar to each other (Figure 52). Nevertheless, a slight decreased of Fv/Fm value was observed in line 4 after 24 hours, but had increased again at day-6 and became stable as in the BIQ wastewater media. In general, *A. obliquus* cells were fit during the experiment and only a slight decrease in line 4 was observed before the start of the flushing mechanism.

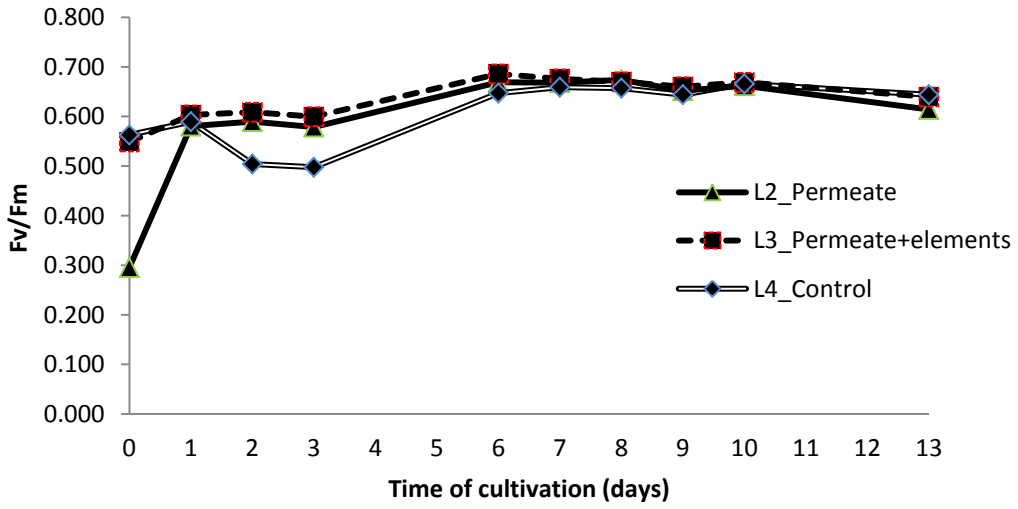


Figure 52. Photosynthetic activities of *A. obliquus* in façade bioreactor at BIQ during the low-light experiment

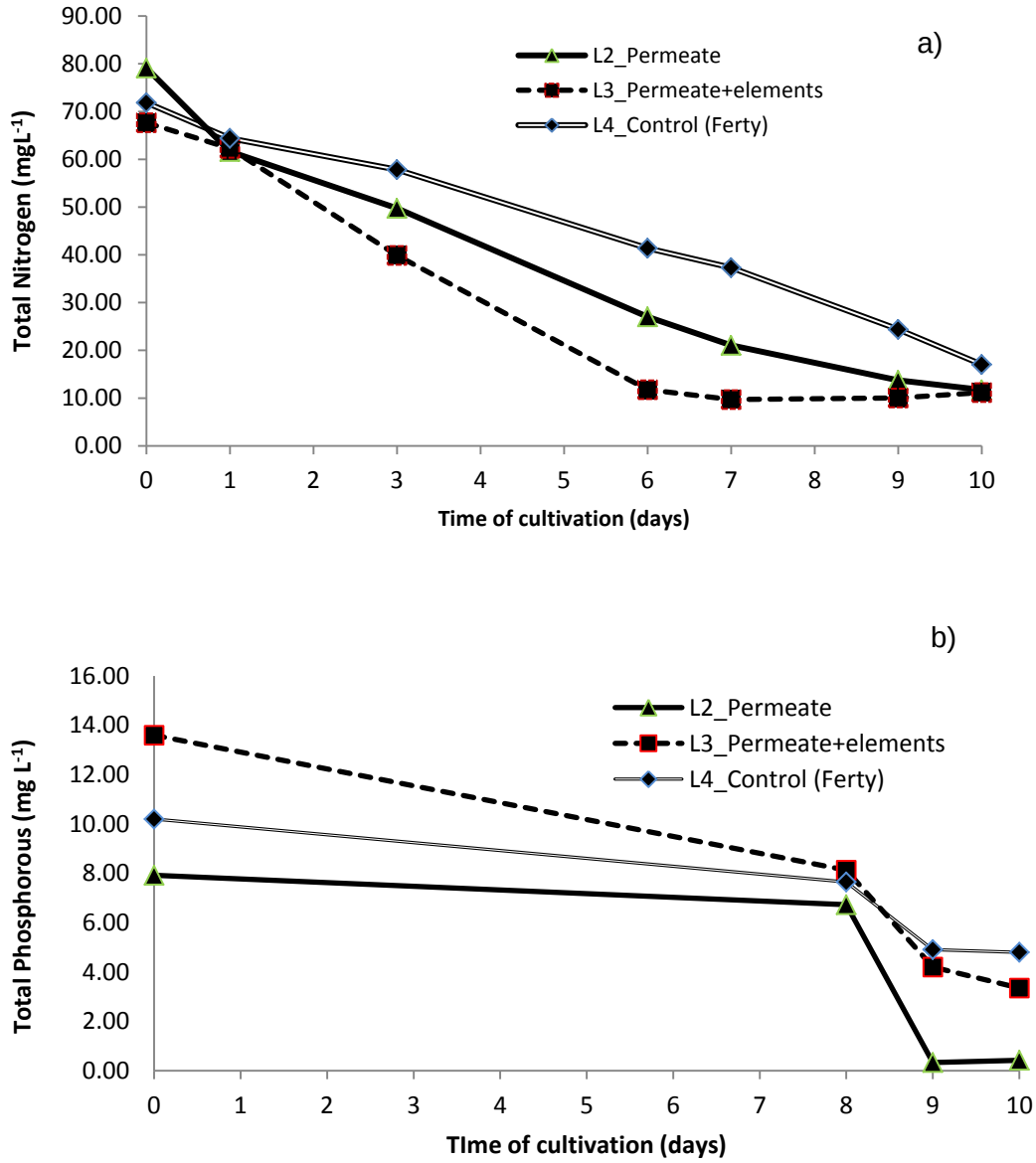


Figure 53. Total Nitrogen (a) and Total Phosphorus (b) concentration in the BIQ photobioreactor façade during the low-light experiment

Total nitrogen concentration decreased during the first 6 days of the experiment (Figure 53-a) although no growth of *A. obliquus* was observed (Figure 51). Unlike total N, significant removal of total P was observed after the flushing system began (Figure 53-b). Percent removal of TN and TP were successively decreased from L2, L3 to L4 (Table 43).

Table 43. Total Nitrogen and Total Phosphorus removal from BIQ wastewater after *A. obliquus* cultivation at BIQ facade photobioreactor during the low-light experiment

	Total Nitrogen (%)	Total Phosphorus (%)
L2_Permeate	85	94.67
L3_Permeate+elements	83	75.37
L4_Ferty	76	52.94

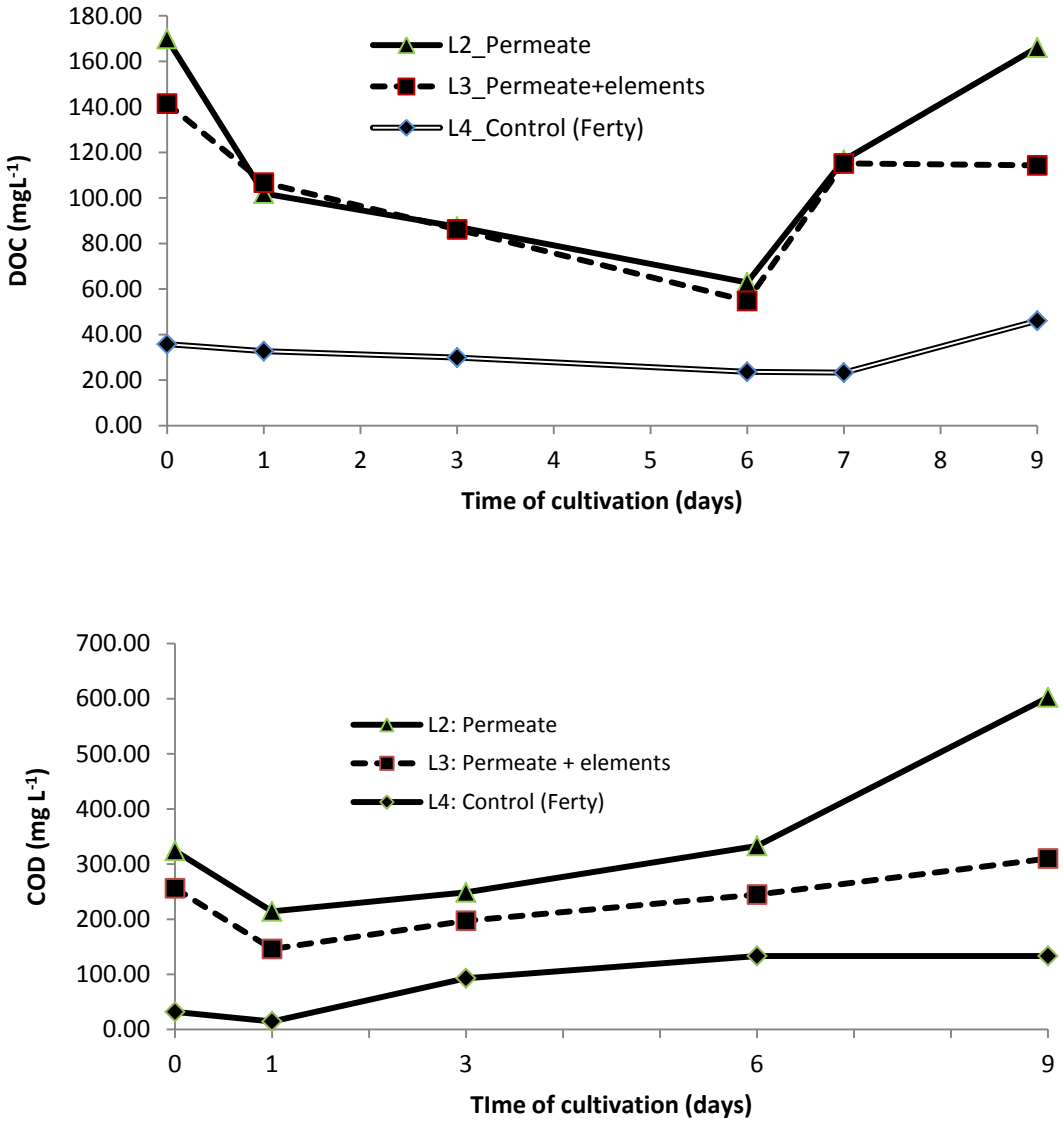


Figure 54. Dissolved Organic Carbon and Chemically Oxygen Demand concentration in the media of BIQ wastewater and in control medium during the low-light experiment

In the first 6 days of the experiment before the flushing procedure started, DOC concentration in all lines decreased about 63%, 61.3% and 34%, respectively for lines 2,3 and 4 (Figure 54). This is concomitant to stagnation of *A. obliquus* growth (Figure 51). In general, this pattern was similar to that of the COD concentration, especially in the BIQ wastewater media, in which the DOC and COD concentration at day-9 was higher than at day-6 after the flushing system was initiated.

4. Discussion

4.1. Laboratory-scale experiments

4.1.1. Cultivation in black water

4.1.1.1. Characteristics of anaerobic-digested black water

In a complete anaerobic digestion process, pathogens and organic carbon will be removed while energy and beneficial compounds will be recovered (Foresti et al. 2006). Nutrient conservation in the liquid effluent from an anaerobic digestion process was also determined by SHIH (1987). It is explained in more detail that a complete anaerobic digestion process would convert organic N (ON) to NH_4-N (Foresti et al. 2006). Hence, NH_4-N concentration could increase in the effluent. Low TN (75.6%) conserved in this experiment was probably due to N volatilization during sample transportation from Hamburg to Berlin, where anaerobic digestion took place. In the experiment by using an Up-flow Anaerobic Sludge Blanket (UASB) reactor, Graaff (2010) conserved 94.73% of TN after 518 days of anaerobic digestion process.

Although Graaff (2010) found that 59% of total phosphorus (TP) was conserved in his experiment, lower amounts were conserved in this experiment (35%), probably due to the filtration after the anaerobic digestion process. Phosphorus is possibly attached to larger particles, especially in its organic or condensed form (Haygarth et al. 1997). Therefore, when more particles were retained on the filter, less phosphorus content was observed in the permeate. However, the filtration process applied in this experiment was necessary to reduce pathogens and particulates that could hinder light penetration within culture.

The nitrogen (N) to phosphorus (P) ratio found in the black water used in this experiment was 21.17, which was 1.32 times higher than the Redfield N:P ratio of 16 (Geider and La Roche 2002). Hence, it was expected that P would become the limited nutrient to support *A. obliquus* growth in this black water, although the results showed that this was not the case.

4.1.1.2. Optimal growth concentration

In this experiment, growth initiation in day-8 (Figure 10) showed element limitation in the anaerobic digested black water to support optimal growth of *A. obliquus*. Composition of elements from Kessler and Cyzgan medium (Kessler, E. und Czygan 1970) were used as a control due to the possibility of N and P separating from other elements.

Based on the productivity values (Table 10) and growth diagram, where no stationary phase was achieved (Figure 10), the highest content of black water of 34.33% was found to best support the optimal growth of *A. obliquus*. Likewise, with additional elements, productivity of *A. obliquus* in 34.33% of the black water was not statistically different than that in the control. Similar productivity (0.34 g L^{-1} d^{-1}) was observed in *Scenedesmus obliquus* which grew in 100% anaerobic-digested black water (Fernandes et al. 2015). In this case, the brownish color of the

black water had no adverse impacts, such as light limitation, to support the growth of *A. obliquus*. In their experiment, Fernandes et al. (2015) observed lower light attenuation coefficient (70-140 m^{-1}) in anaerobic digestion of black water than in a typical artificial outdoor algae culture (200-2000 m^{-1}). This condition intended that most light be absorbed by algal particles than by suspended solids in black water.

The lower photosynthetic values of *A.obliquus* in black water media than in control from day-0 until day-8 of the experiment (Figure 11 a) were attributed to elemental limitation. It is already known that algal growth depends not only on the availability of macronutrients, such as carbon, nitrogen, phosphorus and silicate but also on ions (Na^+, K^+, Mg^+, etc.) and microelements (Fe, Mn, Zn, Co, Cu, etc.). Several metabolic pathways of photosynthesis require those elements, such as iron, which is essential to facilitate photosynthetic electron transport. Likewise, manganese is a critical element in the water oxidizing center of the photosynthetic system (Andersen 2005).

Increasing pH in the media culture of 34.33% BW and in the control was related to the increase of biomass after addition of elements (Figure 11 b). In contrast, lower pH was observed in 11.4% BW and 5% BW. The increased of pH during alga growth is explained below (modified from Chi et al. (2011)) :

In water, dissolved carbon dioxide will be converted into carbonic acid:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \quad (1)$$

The carbonic acid then dissociates into bicarbonate and proton:

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad (2)$$

With the help of carbonic anhydrase (CA), carbon is up taken into the cell. During the conversion of bicarbonate to carbon dioxide, protons are consumed that come from the dissociation of water or are excreted by the cells. Subsequently, hydroxide ions are produced also within the cells. The hydroxide ions in the cell must then be neutralized with protons from the extracellular medium, hence pH of the medium increases. The carbon dioxide is further fixed by RubisCO for photosynthesis.

4.1.1.3. Limitation of elements

4.1.1.3.1. Growth of *A. obliquus* in black water

Compared to black water samples from other studies (Palmquist and Hanæus 2005; Brandes 1978), the magnesium concentration in the black water used in this experiment (6.7 mg L^{-1}) was in the range of that of the other black water samples (4-17 mg L^{-1}) (Table 11). However, iron (0.12 mg L^{-1}) and manganese concentration (0.02 mg L^{-1}) was only about 10% and 15.4%, respectively, of those in other black water samples (1.2-1.28 mg L^{-1} for iron and 0.13 mg L^{-1} for manganese). The lower concentration of elements was probably due to precipitation during the anaerobic digestion process. Since a complete anaerobic digestion process occurred

with methanogenesis as the final process, a normal pH would be observed (de Mes et al. 2003). In normal pH conditions, elements are normally incorporated in solid particles; as such, an acidic condition should be provided for precipitation to be avoided (Marchioretto 2003).

Lower element concentration, which was observed in the black water than in the control medium, was assumed as the limited elements missing for optimal growth of *A. obliquus*. From medium A (without iron) and medium D (where no additional elements were added), no significant difference was observed in productivity (Table 12). Therefore, at first, only iron was assumed to be the most limiting factor to support growth of *A. obliquus* in such black water. However, fast achievement of the stationary phase in the absence of magnesium in medium B suggested that magnesium was another limited element in black water (Figure 12). Nevertheless, only medium C showed that additional iron together with manganese and magnesium properly supported optimal growth of *A. obliquus* in the black water in this experiment.

Iron limitation was also observed by the decrease of photosynthetic activity in media A and D, where the Fv/Fm declined from day-1 of the experiment in both media (Figure 13). It is known that iron (Fe) is one of the most critical elements in photosynthesis. The main role of iron in photosynthesis is as a facilitator of electron transport in the electron transport chain (Heldt 2005; Rout and Sahoo 2015). Raven et al. (1999) mentioned the role of iron in an electron linear transport from H_2O to $NADP^+$. Magnesium is the central atom in the tetrapyrrole ring of chlorophyll, as iron is the central atom of the tetrapyrrole ring of a cytochrome (Heldt 2005). There are 2-3 iron atoms in photosystem II; 5 iron atoms in cytochrome b6-f; 12 iron atoms in photosystem I and 2 iron atoms in ferredoxin (Raven et al. 1999; Rout and Sahoo 2015). As a consequence, changes in the redox-status of iron in at least in one of the protein system in a photosynthesis apparatus may disturb the whole electron transport chain, as fewer atoms will facilitate the process. Observing the growth of cyanobacteria, Wurtsbaugh and Horne (1983) detected that low nitrogen uptake initiated growth reduction when low iron concentration was present.

The achievement of the stationary phase in experiment B at day-12 (Figure 12) was probably a result of the decreased of photosynthetic activity since day-6 of the experiment (Figure 13). Magnesium deficiency is known to interfere the development of chlorophyll a. Tetrapyrrole, the basic structure of a chlorophyll contains 4 pyrrole compounds where magnesium is located in the central part of its structure (Figure 55). As the central atom of the chlorophyll a structure, the role of magnesium in photosynthesis process is very important. Aside from its function as coordinating ion, magnesium is required in the chlorophyll molecule for capturing photons and converting them to adenosine triphosphate (ATP) (Reinhart 1988).

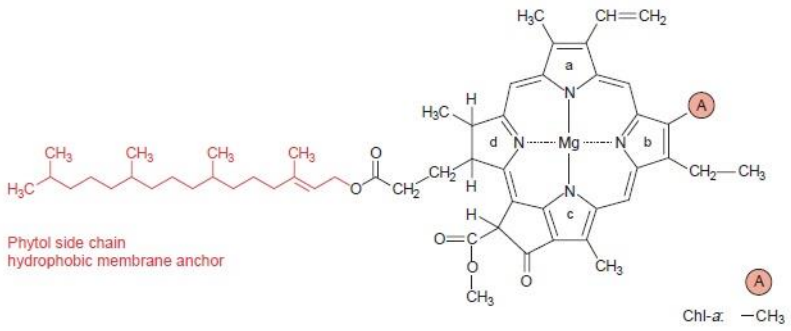


Figure 55. Basic structure of a chlorophyll a molecule with Mg as the central atom (modified from Heldt, 2005)

Manganese was at first considered limited due to its low concentration in the black water (only 14.3% compared to the control media). According to the limited amount of fermented and filtered black water sample, it was decided to add manganese in the media A, B and C to prevent a lack of electron supply (generated by water splitting) to the photosynthesis process. During photosynthesis, light energy received by the chlorophyll molecules in the light harvesting complex is subsequently transferred to the chlorophyll a molecule (P_{680}) in the reaction center of photosystem II. P_{680} will be excited and ionized and the liberated electron is transferred to the primary acceptor. The needs of 4 electrons, cause of one O_2 molecule produced, will be received from the Mn cluster via tyrosine and the deficit electrons in the Mn cluster will be replaced by the electrons produced in the water oxidation process. Hence, the 4 manganese (Mn) ions in the Mn cluster function as a redox compound, which gain and liberate electrons from water. However, the gradual oxidation of water could develop oxygen radicals, which could harm cellular molecules. Thus, manganese cluster will therefore reduce the harmful effect by sequentially supplying the electrons via tyrosine (different oxidation states) to the reaction center (Figure 56). Consequently, less manganese will unable to supply the deficit electrons in the reaction center, harmful oxygen radicals may develop and less oxygen will be liberated. In photosystem II, manganese cluster is also quantitatively considered to occupy the second role after iron in a thylakoid system because of the 4 Mn (and 1 Calcium) per photosystem II involved in O_2 evolution (Raven et al. 1999). Unexpectedly, the addition of manganese and magnesium to medium A had no substantial impact to support the growth of *A. obliquus*, although higher productivity was observed in medium B where Mn and Fe were added. Hence, one could consider that no sole element, but a combination of elements is necessary to support algal growth.

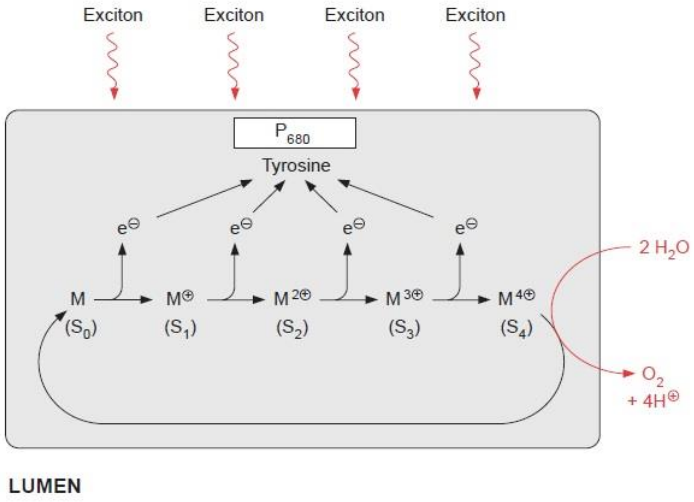


Figure 56. A water splitting process in photosystem II (Heldt 2005). M: manganese in different oxidation states (S0-S4)

Higher productivity of 1.2 times was observed in medium C (where Mg, Mn and Fe were added to BW with) than in medium E (where all necessary elements were supplied). In addition to elements, one difference between black water and artificial medium is the humic substance that causes the dark color of the black water. It was expected that the substance would negatively affect growth of *A. obliquus* but the data showed the opposite. Productivity of *Scenedesmus acutus* in humic substance derived from coal was 1.3 time higher than that grown in an ordinary medium. In this case, the humic substance acted as a growth-regulating substance, which corresponded to auxin or IAA (Indole-3-Acetic Acid) (Pouneva 2005).

Compared to other experiments using similar anaerobic digested black water without filtration, a similar biomass production of about 4 g L^{-1} was found after 13 days (Fernandes et al. 2015). Although filtration did not significantly alter biomass production in my experiments, it was necessary to reduce pathogenic health risks.

Various factors would be able to affect photosynthetic activity of an algal cell, such as salinity (Vonshak et al. 1996), temperature (Vonshak et al. 1994) or nutrient concentration (Qi et al. 2013). However, considering the relatively stable conditions during the experiment, the low photosynthetic activity observed in the beginning of this experiment was probably caused by photoinhibition, resulting from low cell density at the beginning of experiment (Figure 13). Following the growth pattern, relatively stable Fv/Fm values were observed in media C and E. This condition can be deduced from two reasons: first, the addition of magnesium, manganese and iron to the black water successfully supported the photosynthetic activity of *A. obliquus* and second, optimal growth resulted in sufficient cell production and thus, due to high cell density light inhibition was avoided. Low photosynthetic activity in a lower density of a cynobacteria culture was also observed by Torzillo et al. (1996).

4.1.1.3.2. Limitation of elements

Responses between nutrient limitations and algal growth can be examined with various approaches. The model of Monod and Droop (1973) is an acknowledged approach for describing nutrient limitations related to algal productivity (Sommer 1991). Monod relates reproduction rate to extracellular nutrient concentration, while Droop relates reproduction rate to intracellular nutrient content ("cell quota"). Both approaches have their own eminences and drawbacks (Kilham and Hecky 1988). Considering data availability and limited access possibility to the analyzer, element concentrations in the media were chosen to observe limitations in this experiment.

From the first analysis shown in Figure 14 A., 0.146 mg L^{-1} of Mn is sufficient to support growth of *A. obliquus* in medium C, where the highest cell dry weight was produced at the end of the experiment (Table 12). However, with the same concentration algae did not grow in medium A, because no Fe was added to the medium. In this case, Fe addition was probably most important. From the third analysis, growth may be limited at Mn concentration of 0.087 mg L^{-1} as visible within medium B (Figure 15) because at this concentration productivity of *A. obliquus* in medium B was also higher than in medium E, similar to that found in medium C (Table 12). In medium B algal growth reached an earlier stationary phase than in the other media also indicating an element limitation. In this case, growth could be limited early by a Mn or Mg limitation. In medium D, Mn was clearly limited because of the minimal Mn concentration of 0.008 mg L^{-1} (Figure 14 A) and the lowest value of productivity was observed (Table 12). The third analysis (Figure 15) demonstrated that growth was limited at Mn concentration of between 0.087-0.114 mg L^{-1}.

Based on the first analysis, iron concentration of 0.162 mg L^{-1} was sufficient to support growth of *A. obliquus* optimally in enriched BW (medium C) while no Fe was removed from the medium (Figure 14 B). However, the higher Fe concentration at the end of the experiment rather than in the beginning (in almost all media) is still debatable. Based on the third analysis of medium E (Figure 15), growth limitation may occur at Fe concentration of 0.082 mg L^{-1}.

From the first analysis, magnesium concentration of 26.95 mg L^{-1} in medium C at the beginning of experiment supported *A. obliquus* to grow optimally in enriched BW and only 4.2 mg L^{-1} was removed from the medium (Figure 14 C). Growth limitation was observed at Mg concentration of 1.226 mg L^{-1} in medium B, as the stationary phase was reached earlier than for growth in medium C.

The second analysis (Table 13) is based on a calculation of element uptake. However, the amount of elements uptaken was higher than those which were available in the media. The question is how suitable the uptake can be calculated or referred according to the model by Elsayed et al. (2016) and Hecky and Kilham (1988) (sub-chapter 3.1.1.3.2.). The uncertainty was probably due to different conditions between the media in which the alga were cultivated

before (in the experiments of Elsayed et al. 2016; Hecky and Kilham 1988) and the media used for this experiment.

To support complex algae growth, determination of minimal element concentration is required. Calculation of the minimal iron requirement of algae has been shown in marine phytoplankton (Brand 1991). The results present uncertainty and variability due to various factors, such as uncertain iron chemical formations suitable for algae and an adaptation strategy of algae in very low iron concentration by producing biochemical composition that further reduce iron needs.

Based on the results above, experiments with element combinations and the possibility of substance exudation should be further examined. In addition, elements should preferably be analyzed not only from the medium but also from the cell composition.

4.1.1.3.3. Nutrient removal and uptake by algal cells

Through addition of limited elements, *A. obliquus* was able to remove 80.05% of nitrogen from the black water and uptake 78.07% into their cells (Table 14 and Table 15). The lower percentage of TN uptake compared to TN removal was probably caused by N volatilization or decomposition by bacteria. The nitrogen removed from BW was nearly in the same amount as in the artificial medium. In an experiment using the same algal species and the same anaerobic black water without filtration, Fernandes et al. (2015) removed only 27% NH_4-N from the anaerobic black water. A high N/P ratio (of about 30) of the anaerobic black water was used in their experiment, compared to the N/P ratio of 21 used in my experiment. It could be one reason for the higher uptake rates, as an N/P ratio of 21 is more favorable for algae, which typically show an N/P ratio of 16 (Redfield 1958).

Unlike nitrogen, only 53% of total P in black water was removed by *A. obliquus*. This removal was only 56.98% from other findings in anaerobic fermented black water (Fernandes et al. 2015). Like the case with nitrogen, P uptake by the cells (Table 17) was lower than P removal (Table 16). This phenomenon was probably caused by attachment of P to other particles as mentioned by Haygarth et al. (1997) during the measurement, or decomposition by bacteria. Comparing my results to that found by Fernandes et al. (2015), the 1% of P accumulation in the *A. obliquus* cells in their experiment was 5 times higher than the 0.2% of P accumulated in my experiment (Figure 16). Nevertheless, 66.2% of cell dry weight was carbon content in *A. obliquus* growing in medium C. This finding is higher than the 40% of carbon of the dry biomass reported by Fernandes et al. (2015). In my experiment, lower phosphorus and nitrogen accumulation of the cells of *A. obliquus* in medium C has probably generated a higher carbon accumulation (Figure 16). The content of storage products in the cell, such as starch or lipid, is usually produced in stress stages (Tornabene et al. 1983). Interestingly, the highest carbon accumulation in my experiment (medium C) (Figure 16) was accompanied by the highest productivity (Table 12). This phenomenon indicates the advantage of black water as an

excellent growth substrate for *A. obliquus*. The higher carbon accumulation may indicate a switch mechanism from phototrophic to mixotrophic or heterotrophic mode, which was observed by Kim et al. (2007).

4.1.1.3.4. Pigment production

Although identical pigments were produced by *A. obliquus* in different medium compositions, their concentrations varied. At optimal *A. obliquus* growth conditions in black water, cells mostly generated the lowest pigment concentrations. In addition to their function as accessory pigments, carotenoids are produced to protect the cells from photo damage due to light energy overloading the light harvesting complex (Gantt and Cunningham 2001). In my experiments, the highest black water concentration of 34.33% hindered most light penetration and subsequently, low pigment concentration was generated. The lowest chlorophyll concentration occurred at the highest biomass production of *A. obliquus*. Inhibition of chlorophyll synthesis concomitant with increased biomass production was also observed when *Chlorella vulgaris* was cultivated in various organic carbon compositions (Kong et al. 2011). With the availability of organic carbon, algal cells can switch their autotrophic mode to mixotrophic mode. In this case, inorganic carbon uptake was shifted to organic carbon uptake. As a consequence, chlorophyll production may be inhibited while biomass production increased.

Compared to results of *Acutodesmus* isolated from Algerian Sahara (Grama et al. 2014), different kind of carotenoids were generated by *A. obliquus* in this experiment. Among carotenoids, only canthaxanthin produced by *Acutodesmus* was determined by Grama et al. (2014), but not lutein, neoxanthin and violaxanthin. However, production of lutein by *Acutodesmus* is commonly found in various experiments because *Acutodesmus* belongs to one of the most lutein-producing algae (Sánchez et al. 2008). Grama et al. (2014) and Gantt et al. (2001) remarked that different pigment production of the same species could be initiated by different environmental conditions. Besides lutein, neoxanthin and violaxanthin are among the carotenoids which were frequently produced by various species of *Acutodesmus* (Paliwal et al. 2016b). The range of carotenoids concentrations which determined in this experiment were comparable to those found by Paliwal et al. (2016), namely 0.02-4.13, 0.02-0.26, 0.25-2.8 mg g^{-1} CDW, respectively for neoxanthin, violaxanthin and lutein.

4.1.1.3.5. Microstructure of the cells

In all cultivation media, *A. obliquus* showed the resting spore and vegetative forming cells. Various unfavorable conditions could initiate the resting spore cells, such as nutrient (nitrogen) limitation (Kuwata et al. 1993) and aging (Klochkova and Kim 2005). Considering the cultivation time and the remained nitrogen content at the end of the experiment, aging (rather than N limitation) was probably the reason for resting spore cells formation in this experiment.

The lowest percentage of resting spore cells was found in the control medium (medium E, Figure 24). This demonstrates that the control medium has the most favorable conditions for *A. obliquus* growth. Unfavorable conditions in most of the BW media caused a reduction of thylakoid membranes, the disappearance of some inner organelles and the undistinguished boundaries between the organelles. Such conditions were also observed when *A. obliquus* was cultivated in the aqueous phase of a hydrothermal gasification process (Patzelt et al. 2015b). However, such unfavorable conditions in this experiment were also a biotechnological advantage while starch and/or lipid production increased in most of the cells.

Lipid accumulation was observed in the resting spore cells in medium B where magnesium was limited. Initially, it was supposed that an element may effect lipid accumulation or degradation. Depending on the concentration and the compound type added, magnesium addition could be a benefit or drawback for growth and lipid accumulation in algal cells (Kim et al. 2016). In their experiment, increased lipid productivity was observed by adding magnesium aminoclay (MgAC) nanoparticles with concentrations up to 0.05 g L^{-1}. At higher concentrations, addition of MgAC decreased lipid productivity. A certain concentration of magnesium may lead to stressful condition for *Chlorella vulgaris* growth. Furthermore, ROS (Reactive Oxygen Species) were generated but also lead to an increase of the lipid production (Kim et al. 2016). In another experiment with *Chlorella vulgaris*, iron concentration of 1.2 x 10^{-5} mol L^{-1} of $FeCl_3$ caused an increase of lipid accumulation of 3-7 times than that at a lower concentration (Liu et al. 2008). In my experiment, the final Fe concentration of 0.267 mg L^{-1} observed in medium B was only about 40% of the Fe concentration used by Liu et al. (2008) (by conversion from molarity to mg L^{-1}: 1.2 x 10^{-5} mol L^{-1} x 55.85 g mol^{-1} = 0.67 mg L^{-1}. Note: 55.85 is the atomic weight of Fe). Though the concentration was 3.9 times higher than that found in medium D where no lipid accumulation was observed (Figure 15). Also, less lipid accumulation was observed in *A. obliquus* cultivated in medium C, where higher Fe concentration was observed at the end than in medium B. A similar result was described in an experiment with different magnesium concentration (Kim et al. 2016) was found with different manganese supplementation. Additional manganese up to a certain concentration (9 x 10^{-2} µM), slightly increased total lipid production and caused lipid saturation, while at 9 µM lipid production decreased (Constantopoulos 1970). It is difficult to determine the cause of lipid accumulation in my experiment which was based solely on the influence of each element. Lipid accumulation in medium B was probably caused by a combination of supplemented elements. The considerable amount of Fe and Mn that remained at the end of experiment showed that *A. obliquus* growth in medium B could not be limited by both elements. Primarily Mg deficiency occurred which may have caused lipid accumulation. Contrarily, no lipid accumulation was observed in medium D, which was also limited in magnesium. I assumed that magnesium was sufficient for growth until the stationary phase had begun (Figure 12). As such, the cause of lipid accumulation is Mg limitation when Mn and Fe concentration is sufficient, as presented in Figure 15. In addition to

elements, the redirecting of photosynthetically assimilated carbon away from starch synthesis to neutral lipid synthesis may have increased lipid production (Li et al. 2010). In their experiment, inactivation of an enzyme, which activates Glucose-1P by ATP: ADP-Glucose Pyrophosphorylase (AGPase), inhibited starch synthesis and changed the direction of photosynthetically fixed carbon to synthesize neutral lipids, e.g. TAG (Triacylglycerol). Magnesium deficiency increasing the accumulation of total lipids in the green alga *Chlorella vulgaris* was also found in an experiment by Wang et al. (2014). In their experiment, chlorophyll production decreased. In my experiment, chlorophyll concentration in cells of *A. obliquus* in medium B was the second lowest compared to that found in the other media (Table 18).

In medium C, where the highest productivity of *A. obliquus* was observed, lipid vesicles and starch bodies were visible (Figure 22). Compared to the other media, more starch was accumulated by *A. obliquus* in this medium. However, a reduction in thylakoid membranes as well as chlorophyll a concentration was observed. Starch is a carbohydrate accumulation in cells. Some publications mentioned that various environmental stresses would increase the accumulation of starch and lipid in the cells, e.g. high CO_2 concentration, high light intensity and nitrogen starvation (Cheng et al. 2017; Tornabene et al. 1983). In medium C, the highest productivity of *A. obliquus* was accompanied by the accumulation of storage products (particularly starch) and chlorophyll reduction. This was probably be initiated by trophic mode alteration from phototrophic to mixo or heterotrophic, as also observed in an experiment carried out by Kong et al. (2011).

4.1.2. Algae cultivation in the BIQ wastewater

4.1.2.1. Characteristics of the BIQ wastewater

Various factors influence the characteristics of domestic (household) wastewater. Such influences include dietary habits of the people, water usage and socio economic conditions (Meinzinger and Oldenburg 2009). It is very rare to acquire similar information about the characteristics of a mixture of black water and gray water comparable to the wastewater used in this experiment. Most of the information gained from either a mixture of household wastewater and industrial wastewater (Rawat et al. 2010) or source-separated wastewater (where black water was collected separately from gray or yellow water) (Meinzinger and Oldenburg, 2009; Vinnerås et al. 2006).

Compared to similar information of untreated domestic wastewater (Rawat et al. 2010), the TOC, TN and TP concentrations of the mixture of raw BW and Gray Water (GW) used in my experiment were 2.8, 2.6 and 2.8 times higher, respectively, than the highest concentration of such parameters defined by Rawat et al. (2010). According to the definition given by Rawat et al. (2010), domestic wastewater can contain not only household wastewater but also industrial and commercial wastewater. Wastewater addition from industrial and commercial activities may

increase the water content of the domestic wastewater and reduced the concentration of each parameter. Compared to the BW used in the previous experiment, lower concentrations (12.73%, 14%, 12.8% and 23.75%, respectively for TC, TOC, TN and TP) were observed in the BIQ wastewater clearly showing the dilution initiated by the mixing of the concentrated BW and GW. The concentration of magnesium in BIQ wastewater was 1.6 times higher than that in BW. This finding corresponds to the observation in a residential area in Sneek, Netherlands (Hernández Leal et al. 2011). After the anaerobic digestion process, 98% of TN and 96% of TP remained in the medium. This finding was in accordance to other references (Foresti et al. 2006; SHIH 1987).

4.1.2.2. Growth

The element limitation observed in experiments with BW was also found in this experiment. Except magnesium, lower element concentrations occurred in BIQ wastewater than in BW clearly revealing insufficient amount to support optimal growth of *A. obliquus* in BIQ wastewater. The Mg concentration observed in the BIQ wastewater, though higher than in BW, was still lower than in the artificial medium (KC). This was the reason for the considerable supplementation of magnesium in the BIQ wastewater.

An additional chelating agent in one of the media compositions in this experiment was aimed to keep its element availability in the BIQ wastewater for supporting the optimal growth of *A. obliquus*. As has been acknowledged, a chelating agent is required to keep the essential elements in the solution and to preserve the concentration of free elements at a non-toxic level (Andersen 2005). The chelate agent contained in the artificial growth medium, ethylenediaminetetraacetic acid (EDTA), is commonly applied in cleaning agents, such as detergents and soap (Oviedo and Rodríguez 2003). The availability of EDTA, originating from gray water, was most likely to be utilized to preserve elements at such concentrations needed by *A. obliquus* to grow in BIQ wastewater. It was observed that the productivity of *A. obliquus* in BIQ wastewater with EDTA was higher than in BIQ wastewater without EDTA (Table 22). However, KC medium caused lower productivity than the experiment with BIQ wastewater without EDTA. This data indicates that sufficient chelating agent was available in the BIQ wastewater to support *A. obliquus* growth.

In medium C (Figure 26), where no elements are added, the increased of Fv/Fm value after element addition at day-23 showed the importance of such limited elements to support the photosynthetic activity of *A. obliquus*. The significant role of element addition is mentioned in the sub chapter 4.1.1.3.1 above.

4.1.2.3. Limitation of elements

The results of analysis comparing element concentrations in the beginning and at the end of experiment in different media composition (Figure 27) showed that the highest Mn and Fe removal was observed in medium A, where no EDTA was added. Such removal may attribute to lack of chelating agents, so that elements might precipitate and a higher concentration is needed to support the growth of *A. obliquus*. But a higher Mg concentration at the end of the experiment in the BIQ wastewater indicates a sufficient concentration to support algal growth.

The modeled results of element uptake by the cells (Table 23), were similar to that of the BW experiment. Most uptake values are higher than those available in the media at the beginning of the experiment, except for Mg. However, this result is questionable as for the optimal growth in medium B, where 5.89 mg L^{-1} of Mg should have still remained, no removal was observed (Figure 27 C).

In my experiment, growth observed at an Mn concentration of 0.109 mg L^{-1} was limited. This finding is similar to the BW experiment of 0.087-0.114 mg L^{-1} of Mn. This indicated that although only 0.032-0.067 mg L^{-1} of Mn was removed from the media A and B during the experiment, 0.087-0.114 mg L^{-1} had to be provided to avoid growth limitation. In contrast, the highest Fe removal of 0.086 mg L^{-1} in medium A was much higher than the concentration that growth needs to be not limited at 0.042 mg L^{-1} (Figure 28). This concentration is close to the concentration limiting growth in BW of 0.082 mg L^{-1} of Fe. In addition, lower minimal requirements of Fe for optimal biomass production as observed in this experiment would indicate the ability of *A. obliquus* to adapt to such biochemical availability. This phenomenon was also observed when eukaryotic plankton in marine ecosystem showed optimal growth with lower concentrations of Fe than coastal plankton (Eyster et al. 1958). No removal and no indication of growth limitation by Mg indicates that Mg content in the BIQ wastewater was more than needed by *A. obliquus* to grow. This result is supported by the information from the BW experiment, in which growth became limited at Mg concentrations below 1.226 mg L^{-1}. As one of the major elements, magnesium is needed in high quantity. However, the possibility of partial elemental substitution by other elements, such as magnesium with calcium should also be considered (Gerloff and Fishbeck 1969).

4.1.2.4. Nutrient removal and uptake

The N removal of *A. obliquus* in BIQ wastewater (67.4-67.7%) was in the range of N removal by another green alga, *Chlorella vulgaris* (50.8-82.8%), which was cultivated in the effluent and centrate (the wastewater generated in sludge centrifuge) of a municipal wastewater treatment plant in Minnesota, United States (Wang et al. 2010). Although similar N:P ratios were observed (21.17 and 20.59 for BW and BIQ wastewater, respectively), N removal from BW

(80.05%) was higher than in BIQ wastewater (67.4%-67.7%). This was also indicated by the higher productivity observed when *A. obliquus* grew in BW than when it grew in BIQ wastewater (0.357 and 0.213 g L^{-1} d^{-1}, respectively for BW and BIQ wastewater). Nitrogen uptake per gram CDW in the BIQ wastewater with additional elements and EDTA (Table 25) was 1.05 times higher than in BW (Table 15). But in total, N uptake by the cells in the BIQ wastewater was 47.42% of that in BW because lower CDW was observed in the BIQ wastewater than in BW. This finding is in accordance with that found in the percentage of removal. In the BIQ wastewater experiment, the additional EDTA in medium B showed no influence in N removal and uptake compared to that in medium A (Table 24 and Table 25).

Total P uptake in medium B was 1.3 times higher than in medium A (Table 27). This finding shows the influence of additional EDTA in P accumulation into cells. Phosphorus uptake by algae were driven by cell-generated energy, particularly ATP (Jansson 1988; Cembella et al. 1984). Since elements play an important role in electron transport of light reaction in photosynthesis to produce ATP, additional EDTA, which maintains element availability in solution, will further influence P uptake and accumulation in the cell.

4.1.2.5. Pigment content in algal cells cultivated in the BIQ wastewater

The same pigments were produced by *A. obliquus* cultivated in BW, control (KC), and BIQ media. No influence of media composition was visible in pigment production. Nevertheless, higher amount of carotenoids are produced by *A. obliquus* in KC medium than in the BIQ wastewater may indicate an amelioration of photoinhibition mechanism, as explained by Gantt and Cunningham (2001). Although BIQ medium was filtered and exposed to UV light for sterilization, higher turbidity of this medium than KC was visible after 25 days of cultivation. Hence, more light penetrated the KC medium which might initiate higher caretonoid production.

Chlorophyll concentration observed in BIQ wastewater with additional elements was lower than that in KC medium. This finding indicates a switch mechanism from the phototrophic to mixotrophic or heterotrophic mode of *A. obliquus*, due to the availability of organic carbon in the BIQ medium. Unfortunately, chlorophyll concentration observed in the BIQ wastewater without addition of elements (medium C) cannot be compared because pigment content of *A. obliquus* in medium C was not measured in the same stationary phase as in media A, B and D. The decreased of chlorophyll production in the mixotrophic mode of green algae was also remarked by other experiments (Smith et al. 2015; Grama et al. 2016). The ability of *A. obliquus* to grow mixotrophically in wastewater would be an advantage, while growth still continued in mixotrophic mode despite photosynthesis being down regulated. Some possibilities were available for chorophyll synthesis in mixotrophic growth. First, the decrease of nutrient availability (mainly N and P), initiates organic carbon uptake and hence, switches the growth mode from phototrophic to mixotrophic (Kim et al. 2007). The other possibility is an inhibition of

chlorophyll synthesis by organic carbon (in the reference of Stadnichuk et al. 1998, was glucose), which then blocked the formation of coproporphyrin III, a tetrapyrolle compound with an important role in chlorophyll synthesis (Stadnichuk et al. 1998). In my experiment, both arguments may be valid, but further analysis is needed for a better conclusion.

4.1.3. Repeated batch experiments with BIQ wastewater

4.1.3.1. Characteristics of the BIQ wastewater

Compared to the characteristics of the previous BIQ wastewater used (Table 20), changes in some parameters were observed (Table 29), especially for TP, TN and TOC, which are of interest. These changes might be attributed to the human activities in the BIQ building which was out of control. Fluctuation of domestic wastewater characteristics was also found in other wastewater sources (Munavalli et al. 2017; Palmquist and Hanæus 2005).

4.1.3.2. Growth of *A. obliquus* in the first repeated batch experiment

With the first refill of BIQ wastewater (media A and B) lower growth rates and productivities than in the control (medium C) were probably caused by element limitation, as no elements were added. However, addition of limited elements (Mg, Mn and Fe) during the second refill period did not show any recovery (Figure 31).

Until the first 3 days of the experiment, about 2 g L^{-1} CDW were produced in the control medium as well as in the BIQ wastewater as sufficient elements were supplied to support optimal growth of *A. obliquus*. An advantage of BIQ wastewater over the control medium was observed during the first 2 days, as more biomass was produced (Figure 31.A.1.). Due to low initial CDW (of 0.07-0.09 gL^{-1}), cells in the control medium were more exposed to high irradiances. As such, light inhibition may have occurred. In contrast, the light absorption of the yellow substance in the BIQ wastewater may have protected cells from light inhibition. As result, higher biomass was produced during the first 2 days.

In biotechnology, repeated batch experiments are performed to increase productivity with various advantages, such as maintaining high cell concentrations and reducing cultivation time (Ganjali Dashti et al. 2014). In my first repeated batch experiment, the effect of such advantages could be only observed in *A. obliquus* cultivated in the control medium (Figure 31, Table 30 and Table 31).

Sterilized BIQ wastewater showed another advantage over unsterilized wastewater, as microbial contamination is supposed to be one cause of lower growth rates and productivity in unsterilized medium (Table 30, Table 31). Despite the benefits of alga-bacteria interaction, such as nutrient degradation, which further supply the N, P and carbon dioxide required for algal growth, bacterial infection of an alga culture could also have adverse effects, such as the

production of extracellular substances that are toxic for algae or positive for bacterial biofilms and thereby hinder light penetration into the reactor (Zhang et al. 2012).

Growth decreased steadily from the first to the third refill was accompanied with a decreased in the photosynthetic activity of algae, particularly when *A. obliquus* was cultivated in the BIQ wastewater (Figure 32). The reduction of Fv/Fm was presumably caused by unfavorable substances in the BIQ wastewater, which slowed down the photosynthetic activity of the cells.

4.1.3.3. Recheck experiment

The recheck experiment conducted with the BIQ wastewater used also in the first repeated batch confirmed that, aside from Mg, Mn and Fe, no other elements have limited the growth of *A. obliquus*, as shown by productivity, specific growth rate and Fv/Fm values (Table 32 and Figure 34). However, productivity and growth rates of *A. obliquus* in BIQ wastewater were lower than in the control medium (Table 32). This result supports my assumption of a growth inhibition due to an unknown substance in the wastewater. As BIQ wastewater was a mixture of black and gray water, the existence of xenobiotic organic compounds (XOCs), which originated from household chemicals such as cleaning detergents, dish-washing liquid and even softeners from leaching pipes should be considered (Eriksson et al. 2003).

4.1.3.4. Growth of *A. obliquus* in the second repeated batch experiment

During my second repeated batch experiment, growth rates and productivity of *A. obliquus* in medium B (with elements added) were higher than that in medium A (without additional elements). These results confirmed the findings of the previous experiment that all necessary elements were already added to the BIQ wastewater in this experiment. The decrease of productivity and growth rates of *A. obliquus* from the first to the third refill in medium A (Table 33 and Table 34) confirmed the results of the first repeated batch experiment. There, insufficient element supply occurred in the pure BIQ medium and thus reduced growth from one refill period to the next. Consequently, reduction of productivity and growth rates in medium A was accompanied by a reduction of Fv/Fm values (Figure 37). In this experiment, the “Ferty” medium could not be used as a control due to its low growth, particularly after the first refill. In this experiment, the productivity and specific growth rates of *A. obliquus* in “Ferty” medium were lower than in the BIQ medium with additional elements. This may be caused by instability of the pH values in the “Ferty” medium (Figure 36). I presume that the lower pH values in medium C are a result of use of distilled water for medium preparation. Distilled water contains little or no calcium at all. Therefore, water alkalinity could not be maintained. A pH of above 7 is indicative of high calcium amounts (Sonune et al. 2015), and thus calcium maintains the pH of the water. In contrary, tap water, as used for other experiments, has plenty of calcium ions.

Removal of TN and TP (Figure 38) were concurrent with the growth of *A. obliquus* in BIQ wastewater (Figure 35). In another experiment, TN and TP concentration needed to exceed 25 and 2 mgL^{-1}, respectively, to support optimal algal growth (Mostert and Grobbelaar 1987). Contrary to the results of Mostert and Grobbelaar (1987), I still observed exponential growth of *A. obliquus* without any limitation even down to 10 mg L^{-1} and 1 mg L^{-1} of TN and TP (Figure 38). Growth under N and P limitation continues when the trophic mode switches from autotrophic to the mixotrophic or heterotrophic mode, as described by Kim et al. (2007).

A decrease of DOC concentration in every single refill period was observed (Figure 39_A), which followed the increased of alga growth. As a result, the chemical oxygen demand (COD) (Figure 39_B) followed the DOC concentration. Although organic carbon assimilation by algae has been shown by other researchers (Kamjunke and Tittel 2009), the cause of bacterial contamination observed in the *A. obliquus* cultured in BIQ wastewater cannot be neglected. Interestingly, the existence of bacterial contamination, which can be assumed from decreased of DOC concentration and from microscope investigations (Figure 40), showed no inhibiting effect on the growth of *A. obliquus*. Alga-bacteria interactions may benefit or be detrimental to growth of algae (Zhang et al. 2012; Guo and Tong 2014). The interaction in my experiment is attributed to a mutual benefit. Among the mutually beneficial alga-bacteria interactions are O_2/CO_2 exchange and bacterial synthesis of growth-promoting substances (Guo and Tong 2014).

Stable low concentrations of less than 10 mg L^{-1} for TN and less than 1 mg L^{-1} for TP, as observed in medium B during 3 refill periods (Figure 38), fulfilling the regulation for discharging wastewater to the environment (EEC Council 1991). In this case, aside from optimal growth achievement, the addition of limited elements convincingly supported nutrient removal. The final value of COD concentration at each refill period in medium B was less than 100 mg L^{-1}, except at the second refill, where 159 mg L^{-1} remained (Figure 39). In this case, an unfavorable compound might have appeared in the wastewater hindering the cleaning process.

4.1.4. Trophic mode experiment

Various assimilation pathways of various organic substances are performed by algae (Perez-Garcia et al. 2011). A high concentration of organic carbon, as determined in the BIQ wastewater, would probably be an advantageous if such an organic compound was suitable for assimilation by *A. obliquus*.

The highest productivity was observed in trophic mode A (mixotrophic) (Table 39), which may be explained by using organic and inorganic carbon. In mixotrophic growth, the algal cell is more flexible according to its ability to simultaneously collect carbon and energy from organic or inorganic carbon and light sources (Chen et al. 2011). In addition, another advantage is that light is not obligatory in this mode. During mixotrophic growth, inorganic carbon is uptaken due

to the photosynthesis process, while organic carbon is assimilated for respiration. In the respiration process, oxygen supply is compulsory (Perez-Garcia and Bashan 2015).

Trophic mode B was designed to observe the ability of organic carbon assimilation from the BIQ wastewater to support growth of *A. obliquus* without inorganic carbon supply. Acetic acid is mentioned as one possible substance produced in an anaerobic process (Bajpai 2017). Hence, a possible organic carbon source in BIQ wastewater was first assumed to be acetic acid. Productivity in trophic mode B was higher than in trophic mode C (Table 39). The different composition of organic carbon content in trophic mode B may better support growth of *A. obliquus* than organic carbon composition in trophic mode C. The start of exponential growth after day-5 indicated the limitation of inorganic carbon at the beginning of the experiment in trophic mode B (Figure 41). I assumed that during the first 5 days, *A. obliquus* adapted to an unknown organic substance in the BIQ wastewater and initially attempted to grow phototrophically. After the adaptation phase, *A. obliquus* changed the trophic mode and began to grow photoheterotrophically (Figure 41). Hence, an unknown organic substance in the BIQ wastewater was utilized by the algae. Nevertheless, trophic mode D (heterotrophic) showed that without light and without inorganic carbon supply *A. obliquus* hardly grows in BIQ wastewater.

Until day-5 of the experiment, lower oxygen evolution was observed in trophic mode B and C than in trophic mode A (Figure 43). This may be because in trophic mode B and C photosynthesis was limited by a lack of inorganic carbon supply. After day-5, relatively stable oxygen evolutions observed in trophic mode B and C were accompanied with increased respiration rates. In addition, growth increased was observed in trophic mode B and C after day-5 (Figure 41). As mentioned above, there is a possibility that *A. obliquus* switched its trophic mode from phototrophic to photoheterotrophic in this period. A relatively stable oxygen evolution to respiration rate ratio in trophic mode B and C after day-5 of between 1.1-2.3 (except for 3.7 at day-6 in trophic mode B) indicated a balance between photosynthesis and respiration, as also shown by the photoheterotrophic experiment of Grama et al. (2016). In comparison, the oxygen evolution to respiration rate ratio of *Micractinium inermum* in mixotrophic growth supplied with acetate was about 1-2.4 (Smith et al. 2015). This condition indicated that this alga species was able to solely support photosynthesis by CO_2 production from catabolism, such as in an alga-bacteria biocoenosis. The advantage of the gas exchange between anabolic and catabolic working organisms would be an ability to reduce aeration for inorganic carbon supply thus reducing the cost of algal culture operation.

4.2. Outdoor scale

4.2.1. High-light outdoor experiment

During the high-light outdoor experiment, element limitation of *A. obliquus* growth in BIQ wastewater could not be verified. This obscurity was not only related to the technical problems

but also the limited cultivation time. During the first 3 days of the experiment, when the exponential phase was observed, it was assumed that elements were still sufficient. Previous experiments with BIQ wastewater and BW showed minimal element requirements to support optimal growth of *A. obliquus* were 0.042-0.082, 0.087-0.114 and 1.226 mg L^{-1}, for iron, manganese and magnesium, respectively. These limitations were observed after day-14 of the BW and BIQ wastewater experiments. Calculating the percentage of element uptake from cell dry weight was defined by Elsayed et al. (2016) and Hecky and Kilham (1988), 3.1, 0.49 and 0.047 mg L^{-1} (respectively for magnesium, iron and manganese) had to be supplemented to the media for the highest cell dry weight of 0.78 g L^{-1} in L4 (BIQ wastewater without additional elements). From the availability of elements in the previous BIQ experiment, concentrations of 0.008, 0.022 and 10.4 mg L^{-1} were observed for manganese, iron and magnesium, respectively (Table 21). With the addition of limited elements, it was calculated that 0.117, 0.063 and 11.63 mg L^{-1}, respectively for Mn, Fe and Mg was available in the beginning of experiment. Referring to both calculations (referring to Elsayed et al. (2016) and Hecky and Kilham (1988) and the calculation after the addition of limited elements), only magnesium and manganese were sufficient to support the growth of *A. obliquus* in such wastewater. However, growth of *A. obliquus* in L4 indicated sufficient element supply until day-3 of the experiment. Element storage in the initial algal cell of the added culture and the influence of other factors during wastewater transportation from the digester to the photobioreactor, as an example element solved from the pipeline, should also be considered. A backwash process of water softener (regarding its high concentration of chloride and other ions) could also influence element concentration in water flow through the pipeline (Rhode Island Department of Environmental Management Office of Water Resources 2012). What should also be considered are abundant concentrations of micronutrients in the pre-culture.

Photosynthetic activity of *A. obliquus* was concomitantly high with the growth, with increasing Fv/Fm observed until day-2 of the experiment and a slight decreased observed from day-3 (Figure 47) that might be attributed to an initiation of a stationary phase (Figure 45).

Areal productivity observed in my experiment with BIQ wastewater (5.24-5.88 g m^{-2} d^{-1}) was in the minimal range of such productivities as observed in other mass culture experiment, e.g. for *A. obliquus* cultivated in Flory medium plus ammonium nitrate (5-30 g m^{-2} d^{-1}, including biomass losses during night) (Hindersin et al. 2014).

Compared to L2 (Ferty medium) and L3 (BIQ wastewater with additional elements), lower TN removal rate was observed in L4, which may indicate an important role of elements in nitrogen assimilation. In another experiment, low iron concentrations reduced nitrogen assimilation of *Scenedesmus quadricauda* (Rueler and Ades. 1987). As iron is a component of nitrite reductase and is required in a nitrate reductase enzyme system, iron deficiency was reported to decrease the nitrite reductase level in *Scenedesmus* and furthermore reduce nitrogen assimilation by the cell. In addition, low P removal rate in L4 may have been caused

by a lack of essential limited elements. Phosphorous uptake by an alga cell is an energy demanded process, in which elements are actively transported through the cell membrane. The energy source for this process could be provided from an electron transport chain and ATP (Jansson 1988; Cai et al. 2013). Since elements play an important role in electron transport chain and ATP synthesis, their limitation would reduce the P uptake process. However, no elemental limitation was observed in this experiment. Lower N and P removal in L4 may indicate that growth was not only supported by major nutrients, such as N and P, but also by other substances in the BIQ wastewater.

In my experiment, the concentration of dissolved organic compounds (DOC) concentration was in accordance with the growth of *A. obliquus*, especially in L4 and L2 (Figure 49). As a consequence, the chemical oxygen demand (COD) followed the DOC (Figure 49). Organic carbon excreted by algal cells has been described elsewhere (Lignell 1990; Malinsky-Rushansky and Legrand 1996). Various factors could influence the DOC excretion process of algae. Some algae exudate DOC during photosynthesis, while others exudate during stress conditions, such as limited nutrients or light (Malinsky-Rushansky and Legrand 1996). In my experiment, DOC was exudated from the beginning of the experiment, where no light or nutrient limitation was observed, so this indicates DOC exudation from healthy algal cells during photosynthesis.

4.2.2. Low-light outdoor experiment

More than 50% of measured irradiance in the low light outdoor experiment was below that of the laboratory scale experiment (420 µmol photons m^{-2} s^{-1}), where optimal growth of *A. obliquus* in BIQ wastewater was observed. However, productivity (in the BIQ medium without addition of elements) in this low-light outdoor experiment was 1.15 times higher than in the high-light outdoor experiment. Hence, no light limitation was observed in this experiment. Slight increases in Fv/Fm values until day-6 showed a positive influence of flushing on the photosynthetic activity of *A.obliquus* (Figure 52). However, relatively stable Fv/Fm values showed that algae were under good conditions during the experiment. No growth occurring until day-6 of experiment (Figure 51) clearly shows that flushing is essential to support the mixing of the culture in the photobioreactor. Local circulation, operating in each line but also interconnected, was found to be insufficient to prevent cell settling in the culture. As a consequence, the precipitation of nutrients and the algal cells was observed at the bottom, corner parts of connecting pipelines, and within photobioreactors. However, the flushing of the culture was found to be time consuming and reduce the ability of its automatization. In the experiment of Leupold et al. (2013) physiological activity of algal cells was stimulated by hydrodynamic forces. Such physiological features include growth rate, metabolic production rate and membrane permeability (Merchuk 1991). However, increased biomass production and high

Fv/Fm values after the flushing showed no significant disturbance in cells physiology. In this case, low biomass production before day-6 was presumably caused by precipitation of nutrients and algal cells at the bottom and corner parts of pipelines, as well as within the photobioreactors, as described above.

Equilibrium between ammonium and ammonia in aqueous solutions is influenced by various factors, such as pH or temperature (Körner et al. 2001). The decrease of TN (Figure 53 A) without *A. obliquus* growth until day-6 of experiment was probably caused by the degassing process of ammonia (NH_3) from the BIQ wastewater. Decrease of TP concentration after day-8 (Figure 53 B) was in accordance with the growth of *A. obliquus.* However, since the flushing just began after day-6 and the experiment stopped at the beginning of the stationary phase, the short cultivation period was insufficient to show element limitation of the BIQ wastewater for supporting optimal growth of *A. obliquus*.

As in the high-light experiment, growth of *A. obliquus* in the low light experiment also exudated organic carbon. Fluctuation of DOC in the BIQ wastewater media (L2 and L3) (Figure 54) was in accordance with the growth of the algae (Figure 51). As a result, COD values were related to the DOC values. Hence, algal exudation of organic carbon during growth is attributed to the production of healthy cells during photosynthesis, as determined by Malinsky-Rushansky and Legrand (1996).

Productivity observed in the BIQ wastewater in this experiment (4.45-6.67 g m^{-2} d^{-1}) was in the minimal range of the mass culture experiment described by Hindersin et al. (2014) with fertilizer (Flory medium). However, the highest areal productivity in this experiment (Table 42) was 1.13 times higher than that of the high-light experiment (Table 40). This shows that growth was improved by flushing technique, which was not examined in the high-light experiment before. Other mass cultures with different culture devices and algae observed productivities between 20 and 40 g m^{-2} d^{-1} (Wen et al. 2016; Chaumont 1993). However, the highest productivity in this experiment of 0.334 g L^{-1} d^{-1} (Table 42) was similar to that observed by Fernandes et al. (2015) of 0.34 g L^{-1} d^{-1}, who cultivated *A. obliquus* under a controlled laboratory scale in 300 ml Erlenmeyer flask of anaerobic black water. My first mass culture of *A.obliquus* in an anaerobic digested mixture of black water and gray water in flat panel photobioreactor of 1500 L volume shows very promising results since 0.5 kg d^{-1} of algal biomass was produced (each photobioreactor line was filled with 500 L of culture). Assuredly, operational and technical aspects of such photobioreactors must be further examined.

5. Conclusion

The results of my study met the objectives: 1. To observe the growth limitations of *A. obliquus* in black water (BW) and in BIQ wastewater; 2. To observe physiological performances of *A. obliquus* growing in black water and in BIQ wastewater.

By supplementation of growth limiting elements (mainly Mn, Fe and Mg), optimal growth of *A. obliquus* in diluted black water (34.33%) was observed. Similar growth limitations were observed in BIQ wastewater. Growth was limited below concentrations of 0.087-0.114, 0.042-0.082 and 1.226 mg L^{-1}, respectively for Mn, Fe and Mg. However, to determine element uptake rates by cells, a comprehensive analysis of elements, not only from the media but also in cells, would show better quantitative analysis.

Higher productivity of *A. obliquus* in BW and BIQ wastewater than in control media was accompanied by lower chlorophyll concentration. This phenomenon indicated a trophic mode that switched from a phototroph to photoheterotroph diet. This was confirmed by the result of the trophic mode experiment, which showed the ability of *A. obliquus* to utilize organic carbon in the BIQ wastewater. Likewise, lipid, starch and carbon accumulation in *A. obliquus* cells were observed in BW. Two possible reasons for the switch of the trophic mode are the decrease of nutrient availability (mainly N and P) that initiated organic carbon uptake or the availability of carbon dependent that inhibit the chlorophyll synthesis (Stadnichuk et al. 1998). However, further analysis is needed to reach a better conclusion.

Besides the effect of limited elements, addition of EDTA to the BIQ wastewater increased productivity. On the other hand, compared to the control medium, higher productivity of *A. obliquus* in the BIQ wastewater with addition of elements but without EDTA was observed. This result indicated that sufficient chelating agent maybe available in the BIQ wastewater to support optimal growth of *A. obliquus*.

Compared to all pigments, a relative high lutein concentration in *A. obliquus* cultivated in wastewater was observed. However, it was only 78%-83% of its absolute concentration in cells growing in control media. This result indicates an advantage of using domestic wastewater to produce valuable compounds of *A. obliquus*.

Brownish and yellow substances, in BW and in BIQ wastewater, respectively, had no adverse impacts on growth of *A. obliquus*, such as causing light limitations. Even during the first 2 days of experiment, higher density of *A. obliquus* in BIQ wastewater than in control medium was observed. A possibility is that the light absorption of yellow substances protected cells of low density against photoinhibition during the first 2 days. As a result, higher biomass was produced.

The repeated batch experiment confirmed that Mn, Mg and Fe were the elements limiting growth in wastewater as used in this study. The aim of the repeated batch experiments to

maintain high cell concentration and shorten cultivation time was achieved by adding these elements to the BIQ wastewater.

In outdoor experiments no light limitation of *A. obliquus* growth was observed. The highest productivity observed in this experiment was similar to that observed in another experiment at the laboratory scale using 300 mL Erlenmeyer flask. The outdoor experiment showed very promising results since in 1500 L volume of culture 0.5 kg d^{-1} of algal biomass was produced. Higher productivity was observed in the low light than in the high light experiment without flushing technique. Undoubtedly, operational and technical aspects of the photobioreactor façade need to be further examined.

Aside from increased productivity of algal cells, the addition of limited elements increased TN and TP removal from the wastewater, as well as decreased COD in the repeated batch experiment. This result indicates that the requirements for urban wastewater treatment plant discharge as stipulated by the European Council Directive (1991) were fulfilled.

The concept of recovering energy by growing algae in wastewater is not new. My study shows various advantages of using anaerobic digested BW and BIQ wastewater which was never studied before. However, further quantitative and qualitative analysis of bioenergy potential of *A. obliquus* cultivated in wastewater should be performed. However, as every wastewater has its own characteristic, the appearance of interfering substances must be considered.

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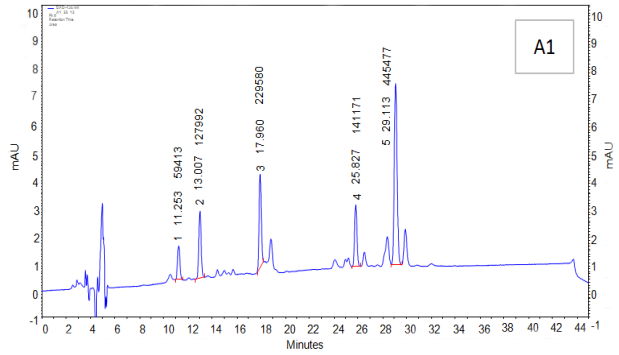
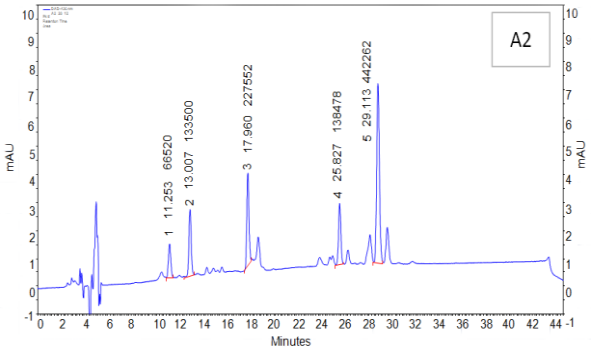
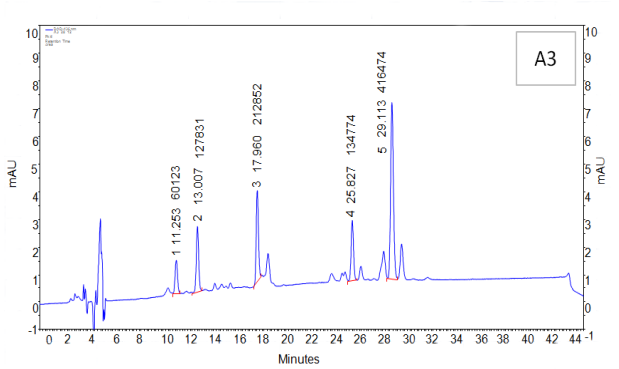
Appendix

Appendix 1. Triplicate data of pigment chromatograms produced by *A.obliquus* cultivated in different composition of Black Water and in a control medium (modified KC)

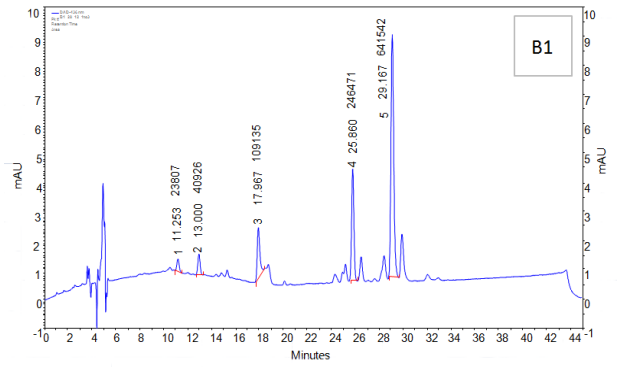
A.

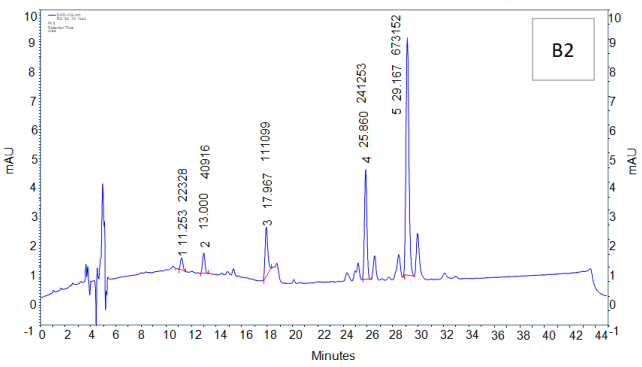


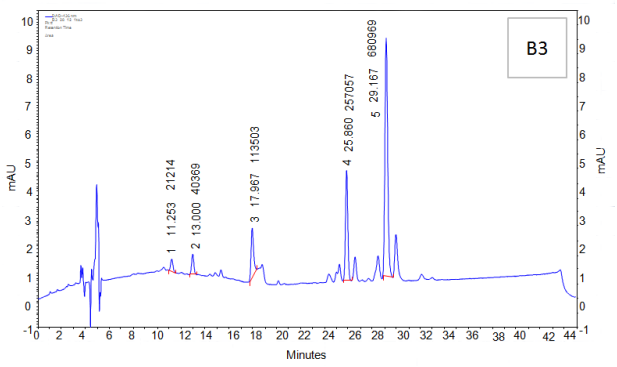




B.



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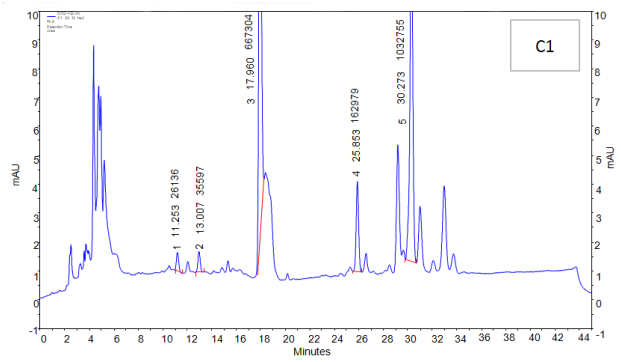
3 17.967 113503
4 25.860 257057
5 29.167 680969

C.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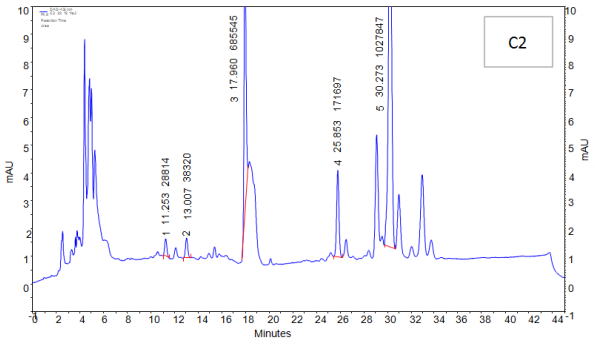
5 30.273 1032755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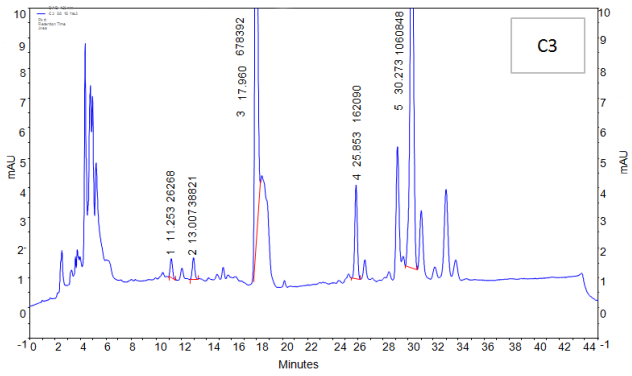
4 25.853 171697
5 30.273 1027847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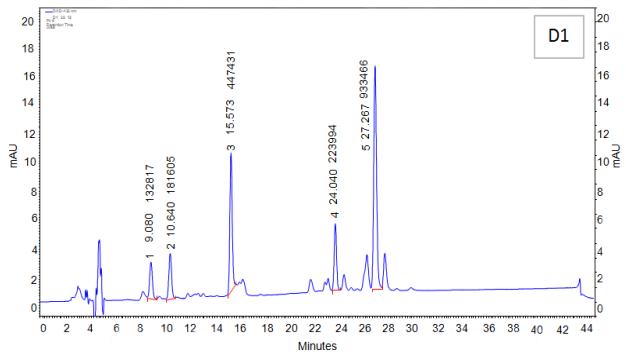
5 30.273 1060848

D.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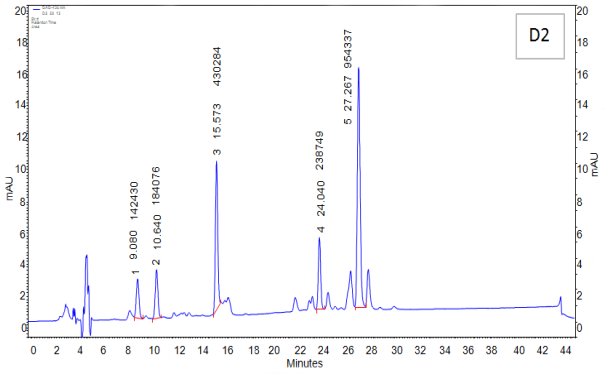
5 27.267 933466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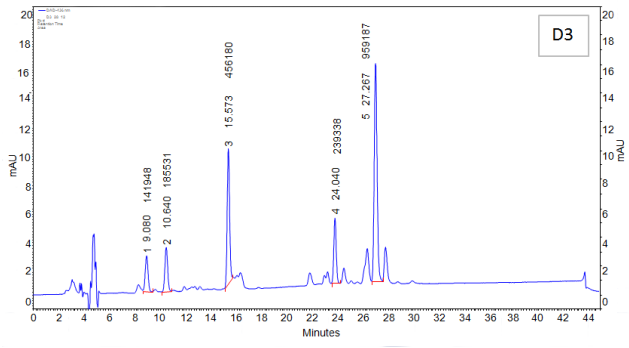
5 27.267 954337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Minutes
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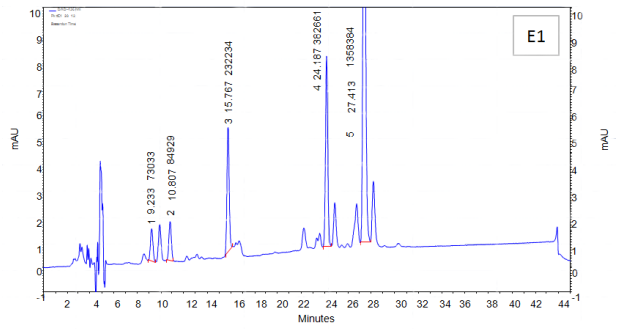
5 27.267 959187

E.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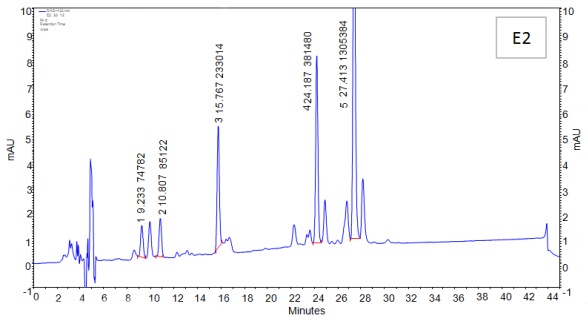
5 27.413 1358364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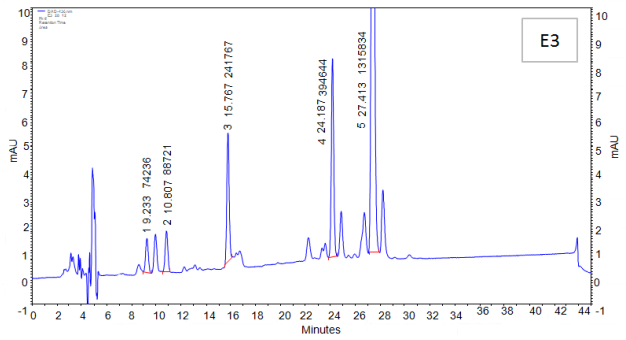
5 27.413 1305384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Minutes
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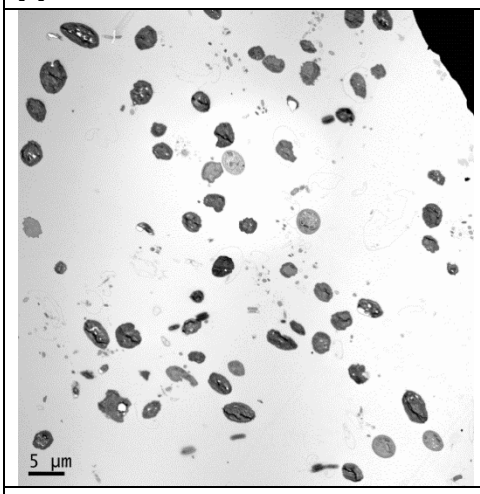
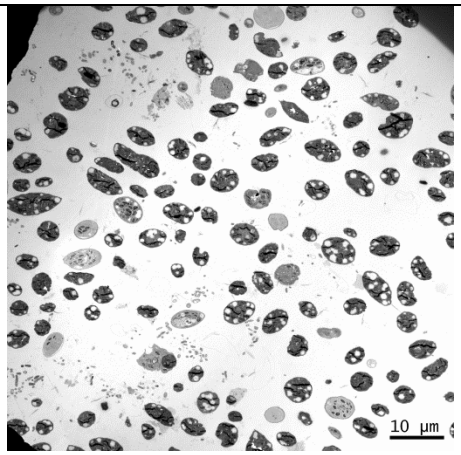
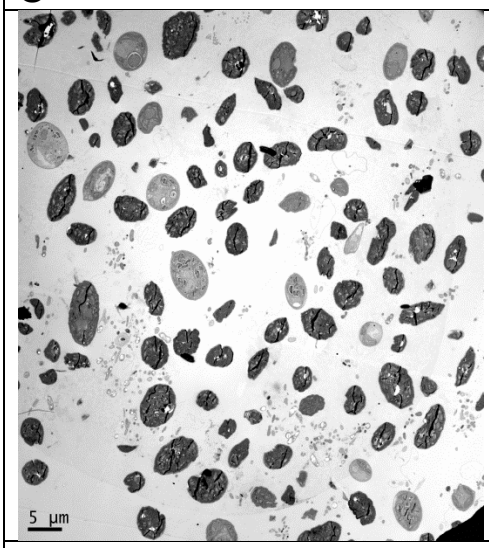
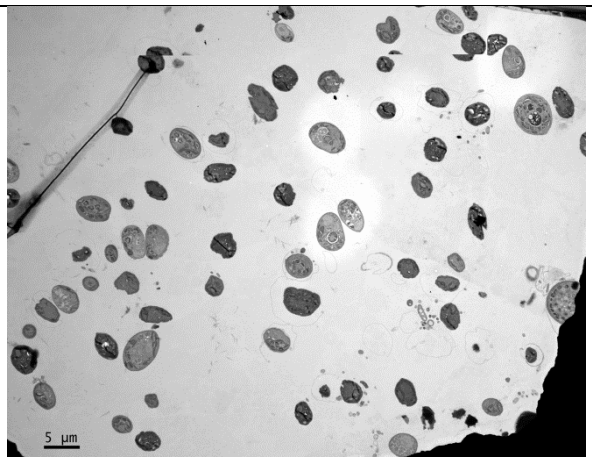
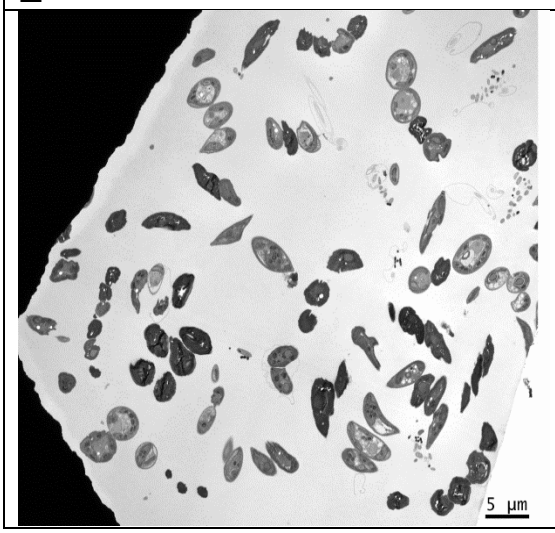
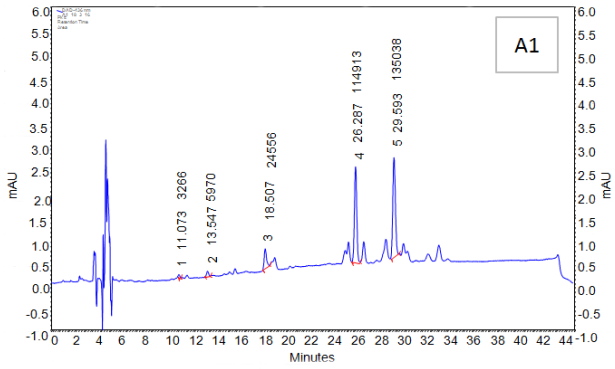
2 10.807 88721
3 15.767 241767
4 24.187 394644
5 27.413 1315834

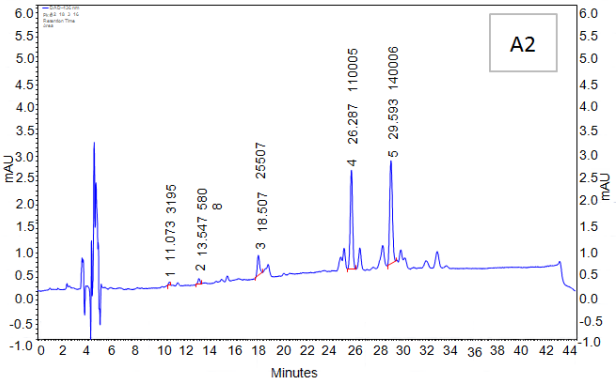
Appendix 2. Transmission Electron Microphotographs of *A.obliquus* cells cultured in Black Water

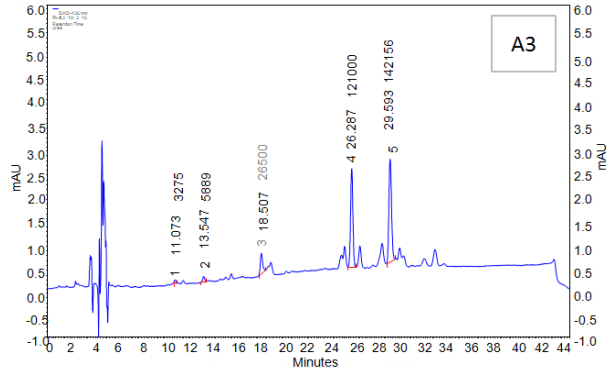
A	**B**
	
C	**D**
	
E	
	

Appendix 3. Triplicate data of pigment chromatograms produced by *A.obliquus* cultivated in BIQ wastewater

A







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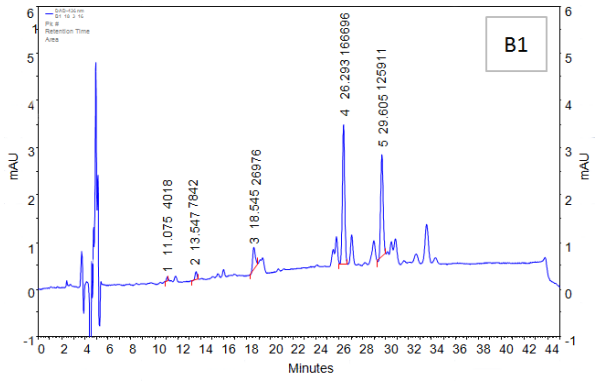

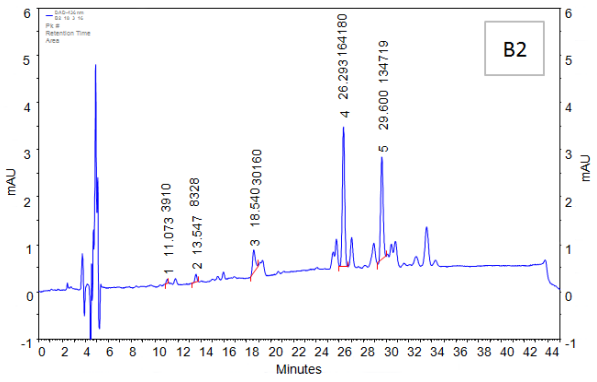

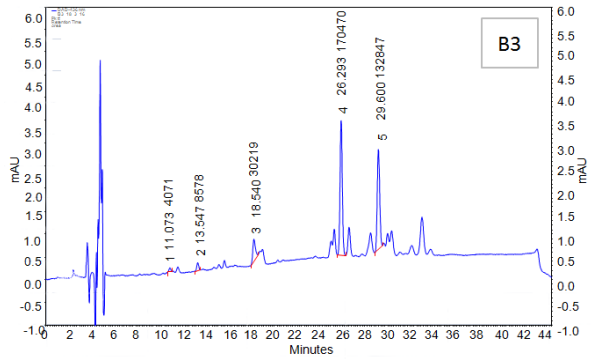

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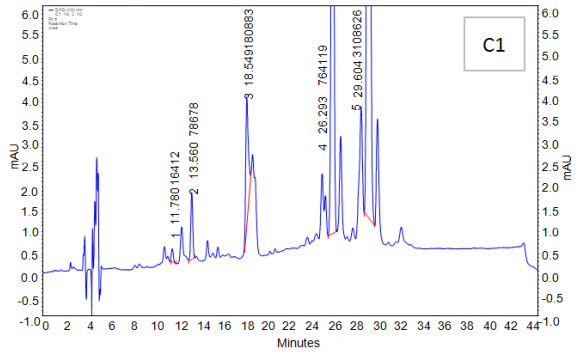
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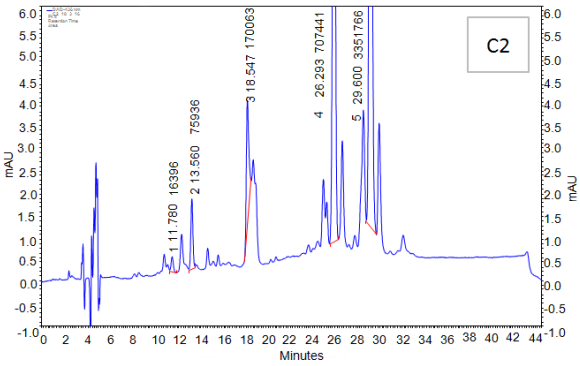
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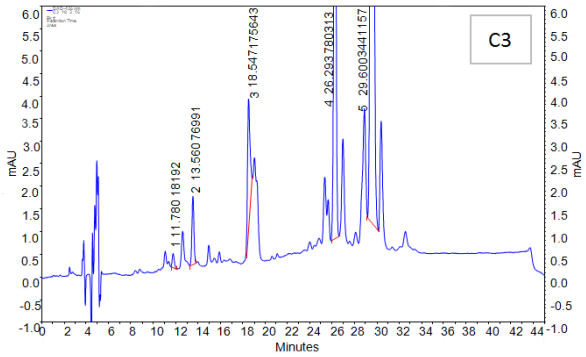
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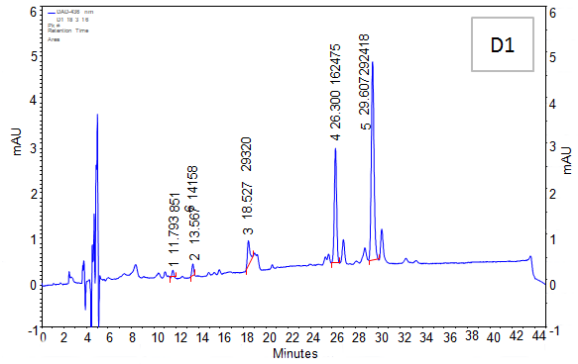
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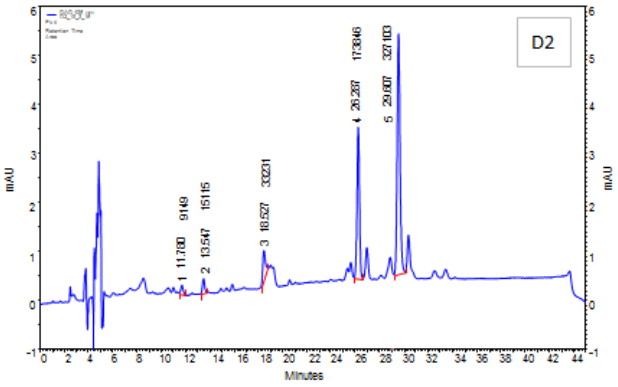
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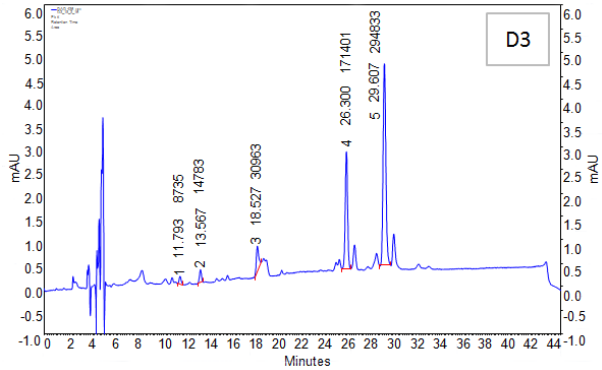
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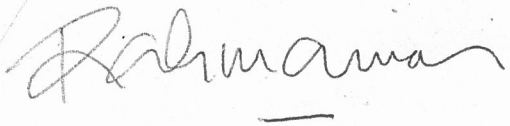
Minutes

Declaration in Lieu of an Oath

I hereby declare that I have written this dissertation on my own and that I have not used other than acknowledged resources and aids.

Hamburg, February 2018



Rahmania Admirasari Darmawan

Universität Hamburg
DER FORSCHUNG | DER LEHRE | DER BILDUNG

cen

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Studienbüro Biologie

To whom it may concern,

I have read the thesis of Rahmania Admirasari Darmawan titled “Growth optimization of Acutodesmus obliquus in anaerobic digested domestic wastewater: advantages and limitations”. In my opinion as a native English speaker, the written English is of sufficient quality to move forward with the evaluation.

Sincerely,

Prof. Dr. Myron A. Peck

Hamburg, Germany 24.01.18

Confirmation of Linguistic Correctness

I hereby declare that I have read the doctoral thesis from Rahmania Admirasari Darmawan titled "Growth Optimization of *Acutodesmus obliquus* in Anaerobic Digested Domestic Wastewater: Advantages and Disadvantages", and I confirm its linguistic correctness in English.

Yogyakarta, 31 January 2018

Christopher A. Woodrich