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Considering human urine as nutrients resource for cultivation of microalgae *Scenedesmus quadricauda* for biodiesel production in photoautotrophic metabolism

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EXECUTIVE SUMMARY

This study aims to illustrate the possibility of using human urine as an alternative to chemical fertilizer and municipal wastewater for the cultivation of microalgae, to produce third generation biodiesel. Human urine has been considered as alternative for other conventional nutrient resource because it is enriched with macro and micro nutrients. In addition, this alternative does not introduce side effects such as being unsustainable, algae contamination and predation with other microorganism available in sewage like chemical fertilizer or municipal wastewater traditionally does. This study has tried to focus on first phase of microalgae biodiesel production which is to increase biomass in nutrients abundance. This is a prerequisite phase for lipid production and accumulation due to nutrients depletion or other sort of biological stresses. In this study, the growth rate of *Scenedesmus quadricauda* has been determined under standard culture (Bold solution) and urine with equivalent phosphorus concentration. The biomass production and chlorophyll generation were considered as indexes of primary production in phototrophic conditions. In all the experiments, diluted human urine performed more efficiently compared to other solutions and is shown as being totally compatible for algae growth and production. The chlorophyll production up to 0.31 mg/L and biomass production up to 64 mg L^{-1} d^{-1} dry mass were achieved. On the other hand, Nitrogen/Phosphorus availability is not well proportioned to promote algal biomass production equally. While phosphorus uptake by algae is over 95%, nitrogen removal cannot reach over 32% in higher urine concentrations which cause high nitrogen levels prevent lipids accumulation due to nitrogen depletion. Urine-based cultures under high levels of phosphorus (as limiting factor) demonstrate growth prohibition in concentrations over 3.0 mg P/L content which can be caused by toxicity of high free ammonia concentrations. Consequently, urine has recognized as reliable sustainable constitution for other nutrient resources for algal biomass production, but the nutrients levels should be manipulated in urine toward enhancing biodiesel production.

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Hadi Soroosh, November 2011

ABBREVIATIONS

Abbreviations	Descriptor
WTP	Water Treatment Plant
CNG	Compressed Natural Gas
GHG	Greenhouse Gas
vvm	Volumes of air per total Volume of bioreactor per Minute
DW	Distilled Water
TW	Tap Water
NB	No Bubbling
HB	Half-Time Bubbling
FB	Full-Time Bubbling
PAR	Photosynthetically Active Radiation
Chl-a	Chlorophyll-A
HU	Human Urine
TAG	Triacylglyceride
FAME	Fatty Acid Methyl Ester
FFA	Free Fatty Acid
ASP	Acid Soluble Polyphosphate
AISP	Acid Insoluble Polyphosphate

CHAPTER 1

INTRODUCTION

There are critical global deficiencies recognised in sustainable development and environmental concerns like carbon emissions, global warming, nutrients and energy shortages and increasing contamination in freshwaters by wastewaters resulting in eutrophication. These issues should be considered through the understanding of linkages of ecosystems which needs to be sustainable. We already experienced the impacts of high CO_2 levels in the atmosphere, with fossil fuels recognised as the major cause of carbon emission [1]. Hence, there is a strong and urgent need to decrease our reliance on petroleum and coal and replace them with renewable and carbon neutral alternative fuels to provide security for energy and environment. Biofuels are one of the sustainable and renewable energy solutions, responding to the energy and climate changes concerns. Biofuels including bioethanol and biodiesel production using terrestrial crops have gained attention in recent decades. However the lack of arable land, competition with the food industry and low yield productivity, caused algae lipids production as third generation of biodiesel to be recognised as the environment friendly and sustainable alternative for carbon fixation and biodiesel production. Algae has shown extensive potential to grow in wastewater which is rich in macro and micronutrients [1][2][3][4]. Using municipal wastewater for algae culture could potentially be facing perils of toxicity and pathogenic infection, in addition to algae loss through predation and competition with other unwanted species and difficulties in harvesting.

Furthermore, food security as a basis of development requires greater attention in regards to water and nutrient supply. Agriculture sector is experiencing increasing deficiencies in nutrient supply. Phosphorus as one of the macronutrients is achievable only by mining and is limited and non-renewable. It is estimated that phosphorus peak could be experienced 2033 by which, demand for phosphorus will exceed the availability of this nutrient [5]. Up to 90% of phosphorus in the world is consumed in the food industry and almost all of the phosphorus in food, excretes by urine and faeces [5]. Phosphorus recovery and recycling from human excreta is inevitable to overcome our current and future global phosphorus shortages. One of the main Ecological Sanitation (ecosan) solutions for this purpose is urine diversion and to use human urine as ecological fertilizer for cultivation [6]. Human urine is the most nutrient rich substance amongst the ecosan products. It contains most of the nutrients available in wastewater and biologically necessary for algal growth. Therefore, urine has the strong potential to be considered as a substitute for wastewater or other artificial algal growing medias. By applying urine for biodiesel algae cultivation, the phosphorus recovery and recycling will takes place practically which is the key point for relying on microalgae production as sustainable biodiesel source. Using urine excludes difficulties due to using domestic wastewater and artificial fertilizers.

Urine can promote microalgal production [7]. Additionally, human urine is cheap and economically viable. This study has followed algal growth with urine and other conventional fertilizers and the possibility of introducing human urine as an efficient growing substance for 3rd generation of biodiesel production under phototrophic conditions.

CHAPTER 2

OBJECTIVES

This study aims to illustrate the possibility of using urine as an alternative to chemical fertilizer for the cultivation of microalgae, to produce third generation biodiesel. Specifically, the target areas in this study are presented as follow:

- To assess efficiency of urine in promoting algae growth in comparison to chemical fertilisers
- To identify the algae reaction to various concentrations of urine in culture media
- To introduce urine as new generation of ecological nutrient source for microalgae cultivation

Overall, this work will attempt to support the utilisation of human urine for sustainable biodiesel production through nutrient recovery in line with ecological sanitation practices.

CHAPTER 3

BACKGROUND INFORMATION

3.1. Biodiesel and Human Urine

Biodiesel as a sustainable biofuel can be produced in different ways. Biodiesel is generally produced from biomass including either terrestrial crops or algae. Regardless of biomass source, nutrients supply for biomass production is essential. Currently different sorts of nutrients are used for biodiesel production. Here we will have a review of different approaches to biodiesel and how urine can be taken into account for biodiesel production.

3.1.1. Biofuel, a New Answer for Global Concerns

Energy is one of the most important factors for social and industrial development. Currently fossil fuels supply the energy for the industries and other civilization demands. However, in recent decades the energy crisis and environmental impacts have challenged conventional energy resources in security and sustainability. Moreover oil and other fossil fuels resources are declining and prices are increasing. Furthermore, oil price is normally affected by wars and crisis in the world which cause petroleum to be unreliable energy source. Therefore, many countries are paying greater attention to alternative clean energy resources and solutions. Nevertheless, the sustainability and reliability of alternative energy sources for fossil fuels like nuclear and hydropower are debatable in light of recent disasters [8][9][10]. Therefore, many countries are turning their attention to renewable energies which can satisfy their energy security and demand. Amongst diverse renewable energies introduced as alternatives for fossil fuel and other unsustainable energies, biofuels are gaining attention and interest globally [11]. Biodiesel, biogas and bioethanol are the most popular biofuels researched regarding their potential productivity and viability.

Additionally, fossil energies are main sources of greenhouse gases (GHG) that contributes to global warming and climate change which are largely generated from industries like fossil fuel-based power plants, natural gas processing, cement, vehicles, steel mass production and waste igniting [12]. We are already experienced the “dangerously high” levels of CO_2 (450 ppm). This highlights to global energy market the urgent need to reduce carbon emission to international legislated target levels [1][13].

Hence, one of the efficient ways to reduce global carbon emission is using biofuels, particularly recycling carbon dioxide by fixing it during photosynthesis process as seen in the production of biodiesel.

> *The most positive impact of biofuels is the reduction of the GHGs emissions in the production and consumption*

Stated Anoop Singh *et al.* 2011, in their article 'Renewable fuels from algae: An answer to debatable land based fuels' [13].

3.1.2. Biodiesel, Next Source of Energy

Biodiesel is an alternative to diesel fuel which is produced from plants neutral lipids via transesterification. While fossil-based diesel is toxic and harmful for environment, biodiesel is biodegradable, more reliable, nontoxic and possibly next primary energy source [14][4]. The base of biodiesel energy is solar power which absorbs and converts to chemical compounds via photosynthesis. Carbon dioxide, sunlight and water are essentials for photosynthesis [15]. In this process carbon sequestrates into micro or macrophytes biomass and lipids as chemical energy which can be derived into biodiesel via transesterification of extracted oil [16]. Therefore any progress to enhance photosynthesis efficiency, for example optimizing light intensity and carbon fixation, improves biofuel yield [1]. Biodiesel as the main photosynthetic biofuel, can be defined as long chain alkyl esters in form of fatty acid methyl ester (FAME), which extracts from transesterification of mono-, di- and tri- acylglycerides (TAG) and esterification of free fatty acids (FFA) resulting a carbon neutral fuel [17][16].

As biodiesel is carbon neutral energy, it can contribute to decreasing carbon monoxide, hydrocarbons, volatile organic compounds and particulate matter emission and also hinders SO_x emanation in atmosphere which are recognized as greenhouse gases (GHGs) and main factors of global warming [14][17]. Biodiesel can be produced from a diversity of corps which have high capacity in carbon fixation and lipid production and storage. Soybean, rapeseed, sunflower, palm, coconut, jatropha, karanja, used fried oil, animal fats and algae considered as main biodiesel resources [14].

3.1.3. Biodiesel Development

Biodiesel production is established upon the productivity and sustainability issues. Generally biodiesel production is categorized into three generations based on development in yield, cultivation, economic and environmental effects and viability. Third generation of biodiesel is recognized as an overriding biodiesel production method. An overview of generations of biodiesel with advantages and disadvantages are shown below:

First generation of biofuels which are produced in large scales, are mainly generated from terrestrial food and oil plants such as rapeseed oil, palm oil, sugarcane, sugar beet, wheat, barley and maize. Producing food crops for biodiesel purposes has caused problems in food production and biodiversity in some countries, ultimately leading to food shortages and high prices. Many first generation biofuels relied on subsidies by governments and are not competitive with conventional fuels like oil. Moreover their contribution in GHG emission is not considered usually. First generation biofuels also require high amounts of water, arable land area and fertilizer for crop cultivation [13][18]. Biodiesel which is derived from rapeseed oil, palm oil or other plants oils is categorised as first generation biofuel.

Second generation biofuels are developed based on shortages and deficiencies in first generation biofuels. The main targets in producing second generation biofuels is maximizing the yield and using non-food based materials such as waste residues, stems, leaves and husks. Second generation biofuels could utilized unused lands thus they are more suitable for developing economics and rural development [19]. The second generation biofuels production can be questionable if it competes with food industry for available land use and also GHG lifecycle reduction contribution.

Third generation biofuels are found in microalgae lipid-rich feedstock which is recognized as the most sustainable and viable alternative for current biofuel [4][20]. Microalgae are uni-cell organisms which use photosynthesis for reproduce and growth. They have relatively high growth rates and energy contents. Some algal strains can double their mass within a few hours with lipid content measuring up to more than 80% of dry mass [15][21]. Third generation of biofuels can be produced on non-arable lands, and this is one of the best advantages the third generation biofuel have over first and second generation biofuels, which food-fuel competition is inevitable. Therefore generating energy from microalgae biodiesel is more economically acceptable [4][13][3]. Biodiesel production from microalgae can reach 15-300 times more than conventional crops. Moreover despite traditional crops which can be harvested only once or twice a year, microalgae can be harvested in 1 to 10 days [1] [22]

Furthermore, wastewater can provide both proper growth medium and free nutrients (especially N and P) availability [1], therefore combination of wastewater treatment (by nutrients removal) and biofuel production is meaningful and beneficial via reducing GHG emissions, energy costs and nutrient and water cost for cultivation [3].

> *Microalgae biodiesel is very competitive as compared to other conventional feedstock in terms of both blue/green water footprint and total water footprint*

[23].

3.1.4. Microalgae Biodiesel and Sustainability

Microalgae biodiesel is a sustainable solution for decreasing stress on resources and reducing air and water contamination. Third generation of biodiesel can meet global and local sustainable development issues relating to nutrients loop and phosphorus recovery, CO_2 emission reduction and carbon neutral fuel production and feasibility. Applying human urine for algae production is a potential solution to increase productivity performance in regards to its sustainability and ecological benefits.

3.1.4.1. Closing the Nutrients Loop

Global phosphorus resources are facing depletion as the main resource for phosphorus is non-renewable mining in limited areas in the world [23]. Phosphorus productivity via mining is decreasing due to less phosphorus percentage and rocks quality. Moreover, the consumption of phosphorus is facing deficiencies in term of fertilizer production performance. Phosphorus consumption efficiency has declined from a mean of 71% in 1995 to 39% in 2003 which means less amount of extracted phosphorus is being converted to fertilizer and more proportion lost in either mining or manufacturing procedure. Furthermore, phosphorus global agriculture sector requires more fertilizer which leads to larger phosphorus mining. For example, high grade phosphorus extraction was 31.67 Tg of P_2O_5 in 2003 which is about 10% of total recognized deposit [24]. It is estimated that phosphorus production peak will take place in 2033 and then the current mineral phosphorus resources would not be enough to meet global phosphorus demands [5].

Additionally, phosphorus can be traced in the food chain. About 100% of the phosphorus which enters into human body is loss via excretion into sewage and wastewater and in many cases enters to water resources leading to eutrophication. Hence current phosphorus lifecycle is not closed because the interrelation between phosphorus non-renewable resources and global demand is not sustainably balanced. Inevitably phosphorus should be recovered from human excreta [6]. Phosphorus removal from wastewater is difficult and phosphorus recovery is not fully possible from precipitated phosphorus in treatment plants. Microalgae have high efficiency for removing phosphorus, nitrogen and trace metals from wastewater. Moreover, wastewater treatment with algae does not generate pollutants in landfills and environment; indeed algal based treatment is potentially an effective remediation method in final nutrients removal in WTPs instead of chemicals and it is a sustainable way to recycle nutrients by reusing Phosphorus and Nitrogen rich by-products [17]. These algae can be treated for producing biodiesel and biogas and the leftover biomass is nutrient rich residue which is perfect for agriculture to make the nutrients loop closed.

3.1.4.2. Reduction of Carbon Emission

Algal cultivation can play an extensive role in carbon emission reduction as it captures large amount of CO_2 (up to 2kg of CO_2 per 1 kg of dry biomass) due to photosynthesis [1][15][17][12]. Most of the main industries like electricity generation, compressed natural gas (CNG) processing, cement and steel manufacturing and urban solid wastes combusting have contributing to emit large amount of CO_2 in the atmosphere. It is because of their reliance on fossil energy sources like coal, petroleum and natural gas [12].

Fixing CO_2 available in flue gases produced from these industries by microalgae for biofuel production is an attractive option for GHG emission reduction although this carbon will be released in atmosphere in burning and consumption process. The point is that carbon credit rises as result of fossil fuel replacement with biodiesel, so in case of biodiesel absence, fossil fuel should be consumed which does not recycle CO_2 in its generation process [25]. Using external flue gases as source of CO_2, make microalgae biodiesel more sustainable and viable as it reduce carbon emission and contributes to the carbon in biomass for further use with very low costs [2].

3.1.4.3. Overall Advantages of 3rd Generation Biodiesel Production

There are plenty of discussions and researches about biodiesel advantages and profits [17]. Clearly, microalgae are the most advantageous biodiesel production so far. The main advantages of third generation biofuel are [1][14][17][4][23]:

1- Higher photosynthesis performance and higher yield productivity because of fast growth rates
2- Can be harvested almost all year round in batch production
3- Able to utilize waste and salty water to save fresh water
4- Combines carbon neutral biofuel production with carbon sequestration
5- The achieved fuel is highly biodegradable and atoxic
6- Microalgal biofuels do not threat food security and hinder food scarcity and high prices
7- Cause less contaminations and GHG emissions in comparison with fossil-based fuels
8- Microalgae can grow on non-arable lands and do not cause land-fuel competition
9- Can take advantage of industrial flue gas as carbon source for photosynthesis
10- Microalgae production does not promote fertilizer use in soil and negates additional N_2O
11- Except biofuel, variety of side products are obtainable from microalgae such as animal feedstock and also pharmaceutical and cosmetic products

3.1.4.4. Feasibility of Microalgae Biodiesel

Algal biofuel has the most potential as renewable energy source for industry and transportation. In addition, microalgae biofuel has less negative impact on environment and food supply in comparison with prior biofuel generations. The most important challenge for third generation biofuels will be lowering the cultivation costs and optimizing the oil productivity [1][13][23]. In this study, the goal has been to increase algae productivity using urine which is cheap instead of expensive and unviable fertilizers to make biodiesel production more feasible and competitive with fossil fuels.

Many studies compared the feasibility of the biofuels and compare them with fossil and other available energy carriers. Biodiesel will be the potential pioneer as a renewable biofuel and primary energy source [26][4][3]. Microalgae biodiesel production, offers side productions which can support its feasibility in order to surplus benefits which are originated from secondary products and advantages next to the biodiesel production process. After oil extraction, the microalgae biomass can be used in industrialized fermenters (biodigesters) to produce biogas and bioethanol which are renewable and ecological energy sources for variety of applications. Biomass Energy Conversion Technologies (BECT) suggests solutions to convert algae biomass after oil extraction into sorts of bioenergies including combustion, pyrolysis, gasification, thermo-chemical liquefaction, biomethanation (Biogas), photobiological hydrogen generation and alcoholic fermentation (Bioethanol). Anaerobic digestion of microalgae biomass (Biogas) next to biodiesel production has been declared as an efficient way to increase biodiesel viability. [26][14][27].

Algal biomass is a protein rich food for animal and human consumption [27][28]. Moreover, the biodigester effluent after biofuel production is a nutrient rich material which consists of macro and micro nutrients and can be considered as fertilizer for either algae production or crops cultivation. This nutrient and energy recovery and recycling demonstrates microalgae biodiesel production as highly sustainable and profitable renewable energy generation [15] (Figure 1).

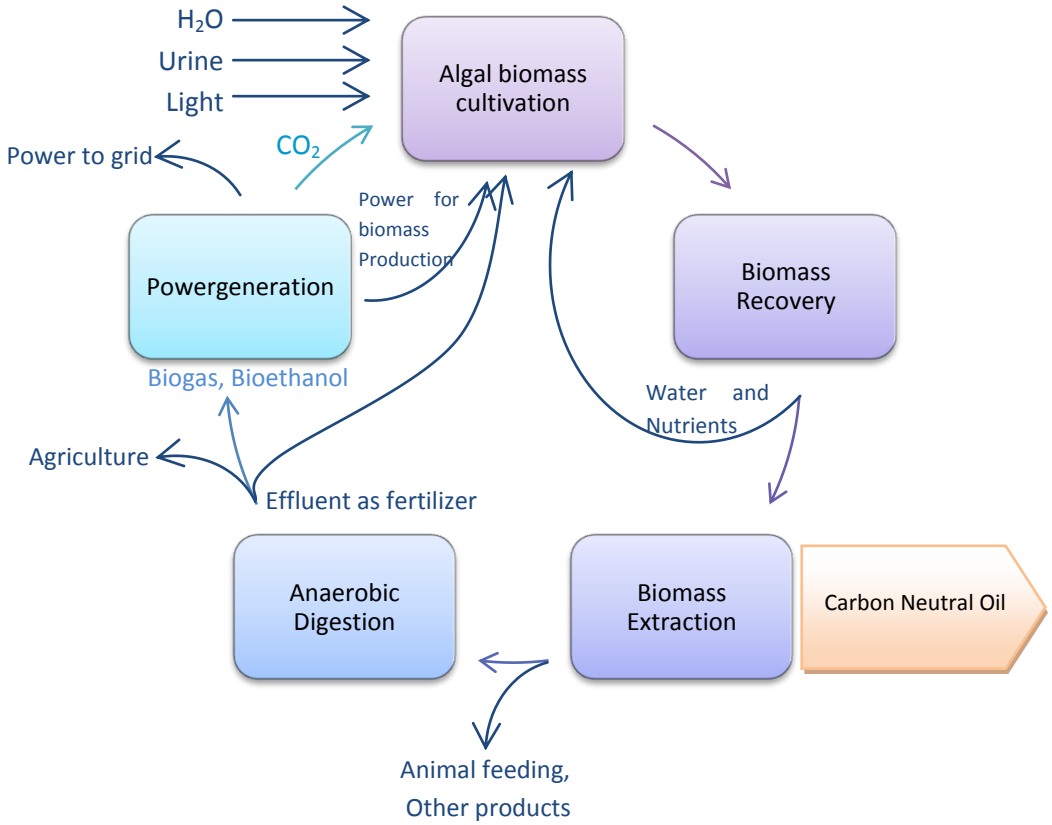

Figure 1- Biofuel sustainable cycle and trends in biotechnology – Derived from [26]

Microalgae biodiesel generation would be viable if massive industrial production is targeted, otherwise the initial and operation costs cannot be covered. It has been suggested that 1.5 to 3 times higher biodiesel production is needed for its economical feasibility [4][11]. One litre biodiesel costs about 50€ which with current prices of fossil fuels is not easily comparable [15] whereas other studies have demonstrated that microalgae production is competitive with petroleum if the price reaches above 67$ per barrel [21] which was done years ago. For reducing the production costs, considering solar energy as an infinite free energy source is inevitable. Essential nutrients for photosynthesis can be obtained from chemical fertilizers or wastewater but these nutrient sources have their disadvantages in terms of feasibility or performance. Human urine is cheap and consists of main nutrients for algal biomass production. Hence, it can play a significant role in reducing the costs and enhancing the viability of microalgae biodiesel.

3.1.5. Using Urine, a New Approach to Biodiesel Production

Choosing a proper nutrient source is an important parameter for microalgae production. Maintaining macro and micro nutrients in the optimum level, results in good biomass yield. Many studies have corroborated wastewater as a sustainable nutrient source for microalgae biodiesel production. Domestic wastewater consists of household and urban sewage and normally has been made up of several different substances and compounds including vital nutrients for microalgae production [3][14]. Nevertheless artificial fertilizers and municipal wastewaters have their respective disadvantages in meeting both technical and economical aspects of biodiesel production simultaneously. Urine includes the advantage of wastewater in term of nutrients availability and sustainability but challenges we have when applying wastewater. Using conventional fertilizer is not comparable with urine regarding nutrients cycling (phosphorus recovery).

3.1.5.1. Wastewater and Problems Regarding to Microalgae Cultivation

Although wastewater can supply required nutrients for microalgae cultivation, it can also restrict algae productivity. Major disadvantages of using wastewater (not source-separated sewage) are mentioned briefly in the following paragraphs.

3.1.5.1.1. Light Inhibition

Usually wastewater is highly turbid because of organic and inorganic fine materials content. High turbidity reduces light penetration in culture medium and causes photo inhibition for reducing photosynthesis. Although the nutrient availability in wastewater is generally sufficient for promoting algae growth, only the shallow layer of culture can reach the required irradiance for autotrophic metabolism which is important for increasing the biomass production.

3.1.5.1.2. Predation, Parasitism and Competition

Zooplankton and unwanted phytoplankton strains which are usually available in wastewater can seriously threat target (lipid-producer) algae species and decrease the yield to very low levels. Rotifers and Cladocerans in high concentrations can decline algae density up to 90% within 2 days. It has been shown that *Daphnia* grazing reduces chlorophyll-a up to 99% within few days [2]. In some preexperiments, Protozoan has shown to make a negative influence on algae growth due to predation (Figure 2)

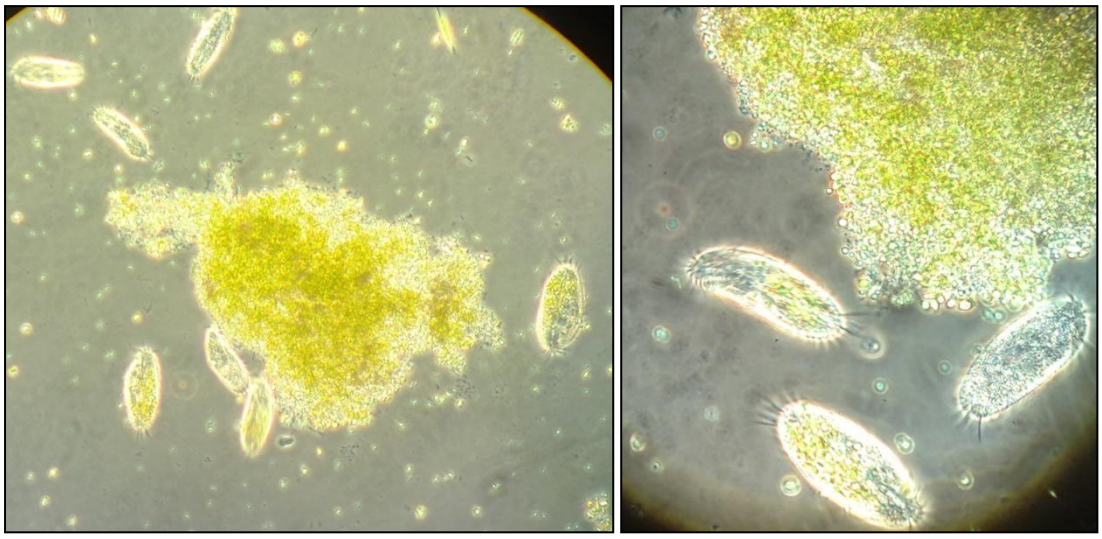

Figure 2- Protozoa predation in water sample from a stream in Kristianstad, Sweden (Soroosh, 2011)

Fungal parasitism and virus and pathogenic infections and contamination by native algae are other threats can endanger population of biodiesel microalgae cultivated in wastewater [2][3] and few chemical solutions are available for controlling the undesirable microorganisms [14].

3.1.5.1.3. Toxicity

Poisonous substances are other major risks in using wastewater for algae cultivation as they can be found in dangerous levels and cause algal mortality and damages. Heavy metals are one of the main toxic contaminants in wastewater. Toxins like cadmium, mercury and zinc in addition to organic compounds which are available in wastewater (especially in industrial sewage) are harmful to algae growth and reproduction [1] [3].

3.1.5.1.4. Hygienic Concerns

The hygienic problems regarding the use of wastewater can cause problems for human and livestock directly and indirectly. Wastewater is a source of pathogenic organisms which can cause serious infections and diseases in human and animals. Using wastewater for biodiesel production and use the left over materials for other side products needs to be hygienically regulated and managed.

3.1.5.1.5. Nutrients Composition

Nutrients in wastewater come from different sources sources like household wastewater (black water), agriculture and gardening runoff and etc. The proportion of nutrients levels in domestic wastewater is variable and proportions of ingredients in wastewater are out of control. Therefore, the nutrients ratios are not constant but microalgae production for biodiesel generation requires highly maintained nutrients availability and proportions to reach the highest possible biomass yield. This problem can be solved by using urine usage which is acceptably reliable in nutrients content and proportions. Nutrient levels when feeding algae with urine can be easily manipulated and adjusted to the optimum level to provide the appropriate condition for biomass production and/or lipid production trade off.

3.1.5.2. Human Urine, a Remarkable Nutrient Resource

Phosphorus availability for agriculture is going to be a critical issue in the future. Phosphorous enters the human food chain as fertilizer and is stored in plant biomass which can be consumed by either animal livestock or humans. Unfortunately, this macronutrient is lost through wastewater and excreta. The excreted phosphorus can cause either eutrophication in water bodies (if it does not receive efficient treatment) or extra costs for phosphorus removal in water treatment plants. For recycling nutrients for sustainable food supply, phosphorus recovery from wastewater is inevitable. Ecological sanitation as a sustainable solution, promotes methods for wastewater separation at source and nutrients recovery. Urine is the primary nutrient-rich compound in domestic wastewater and one of the main products of ecological sanitation practices. Human urine makes up a major proportions of nutrients (N, P and K) in urban wastewater [29][30] (Table 1). Different studies present different ranges of nutrients in urine. Since that the nutrients level in urine dependent on the person's diet. For example, the phosphorus in urine sample within 24 hours is in range of 0.4 to 1.3g for adults but this value is less than 1.0 g for people with calcium and phosphate restricted diet [31]. Phosphorus is taken up by microalgae and is one of essential components in cellular structure and membranes. Phosphorus is necessary for producing biological compounds like nucleotides, phospholipids and nucleic acids [32].

Table 1- Composition of wastewater separation products in household wastewater [30]

Nutrient	Urine	Faeces	Grey water
Nitrogen	10	1.5	1.3
Phosphorus	1	0.5	0.5
Potassium	2.6	0.55	2
COD	15	35	60
Pathogens	Very low	Very high	Moderate

Conventionally urine has been mixed with other wastewaters and is part of blackwater. For achieving urine as the pure, safe and efficient fertilizer, urine separation has been introduced under ecosan concepts. Urine separation is an efficient way for nutrients recovery from wastewater [33][6] and it has been proven using source separated urine as nutrient resource for algal biodiesel production is more environmentally profitable than other terrestrial biofuel plants [3]. Urine diversion is considered as one of the millennium development goals (MDGs) emphasized on its phosphorus and nitrogen content and advantages

“

> *Urine diversion within municipal wastewater jurisdictions is one possibility for reaching the objective of phosphorus recycling.*

”

As mentioned by Tor Borinder, Head of Industry Section, Implementation and Enforcement Department Swedish Environmental Protection Agency [34].

3.2. Microalgae Cultivation

Essential parameters for microalgae cultivation includes proper culture environment, water, nutrients (macro and micro), light and carbon (in CO_2 form) supply. Moreover, for optimizing the lipid and biodiesel production, adjusting and considering other parameters like temperature, mixing, gas distribution, light cycle and intensity, water quality, pH, salinity, cell density and growth prohibition should also be brought into account [1][3][4]. Main conditions affecting microalgae biomass production has been reviewed in following sections for culturing algae in phototrophic metabolism system, targeting the maximizing biomass production.

3.2.1. Culture Environment

Water footprints in microalgae-based biofuel depend on the cultivation type. Basically, there are two algae cultivation systems which consist of open ponds so called high rate algal ponds (HRAPs) and photobioreactors (PBRs). HRAP includes simple open tank or pond which expose suspended algae to atmospheric gases and sunlight via natural contact with surrounding climate. [15]. Photobioreactor is an illuminated culture container (or tube) which is used for maximizing the biomass production through photosynthesis process. There are mainly three types of photobioreactors [35]:

1- Open raceway ponds, are shallow and circular channel systems. For hydraulic purposes, the depth should be more than 20 cm. This photobioreactors normally produce low biomass density (0.3 g DW per litre)

2- Tubular photobioreactors, basically consist of transparent tubes, centrifugal pump and degassing tank. The microalgae circulates in the tubes by pump power and continuously passes through degassing vessel which can lose accumulated oxygen and be conducted to harvesting system.

3- Flat panel photobioreactor, is a flat transparent vessel with gas injection agitation system with rate of 1 litre per 1 litre reactor volume per minute.

Closed bioreactors are more advantageous than open ponds as [23][12] :

- They can produce higher densities of microalgae per volume of water
- Provides more control over growth conditions (e.g. light, temperature, pH and nutrients level)
- They reduce water use due to less evaporation.
- Their performance is not related to fluctuations in temperature and light intensity due to seasonal changes.

Mixing in photobioreactor distributes nutrients evenly and also causes all the cells to get exposed to light [12]. The photobioreactors performances and feasibility varies depending on irradiation conditions, mixing and photosynthetic efficiency of systems, medium- and CO_2 costs [35]. While while some studies do not suggest photobioreactors for biofuel production because of high capital costs and technical restrictions in scaled-up systems implementation [36]. The round containers are the best shape to receive more light and distribute light equally into the vessel [12].

3.2.2. pH

pH has an important role in algae growth rate and phosphorus availability. Biological metabolisms in algae can elevate the pH level in aquatic environments via carbon consumption and oxidation of organic algae wastes. When pH increases, the phosphorus precipitation raises through binding with calcium, iron or aluminium ions [32][2]. Increase in pH, affect CO_2 availability and ionic nutrients and their absorption which are necessary for algal growth and metabolism. High pH values in addition to free ammonia concentration can cause considerable interference in fresh water algae photosynthesis [1][2]. Generally pH level of 8 is optimum for algae growth but some high pH adapted strains (e.g. *Amphora* sp. and *Ankistrodesmus* sp.) can tolerate pH of 9 and even 10 [2].

3.2.3. Temperature

Temperature can assists phosphorus assimilation in algal biomass due to effects on biological activities and also cell constituents. Increase in temperature has shown to affect polyphosphate accumulation and changes in fatty acid composition (fatty acid methyl ester (FAME)), protein concentration and the nitrogen-to-carbon ratio in cellular structure [32][16]. The optimum temperature for enhancing algal growth depends on the algae strain and nutrient availability but range of 28 to 35°C is recommended for many species of algae. [2]. Moreover, temperature affects the ionic balance in culture medium and modifies pH, solubility of CO_2 and oxygen which can bring out different responses in assorted algae species [2]. Abrupt temperature changes, causes intensive reduce in algae productivity [2], therefore, it is important to adjust the temperature to a steady level.

3.2.4. Mixing and Carbon Supply

For maximising photosynthesis, mixing is necessary. Mixing improves nutrient and gases (CO_2 and O_2) distribution and removal, light exposure, cooling the culture medium and preventing algae cell to settle down.[1] There are several methods for mixing and stirring algae culture. The most popular mixing method is bubbling with air or CO_2 injection into the culture. This method maximizes carbon capture and increases the feasibility of whole process by energy costs reduction [37]. The point about mixing by bubbling is extra evaporation which can increase water lost and altering the ions equilibrium and pH in algal medium [15].

Main methods for mixing are mechanical and bubbling. Whereas mechanical mixing can damage cells, air-CO_2 bubbling has been recognized as the most efficient way. The source of carbon dioxide can be flue gasses from power plants and other fuel oxidation based industries and machineries to be more sustainable and feasible [1]. Photosynthesis in high productive ponds and reactor can cause dissolved oxygen level reaches over 200% saturation in daytime. Oxygen saturation in range of 200-300% declines photosynthesis up to 25% therefore CO_2 bubbling can remove the extra dissolved oxygen in the media and can increase the algae cell productivity up to 30% by providing more carbon to be synthesized [2], Moreover CO_2 injection releases H_2CO_3, HCO_3^- and CO_3^{2-} in the culture media. The concentration of each of these substances depends on temperature and pH. But increase in these anions, cause increase in H^+ cations leading to pH be reduced [12]. If the pH be maintained below 8, more nitrogen would be available for algae metabolism and assimilation due to less ammonia volatilization [2].

On the other hand, overloading in CO_2 concentration is undesired. Overfeeding CO_2 causes a decrease in biological CO_2 sequestration capability with microalgae cells and drastic pH drop. Consequently CO_2 concentration should be adjusted. Some studies have demonstrated optimum concentration for CO_2 as 0.25 vvm (volumes of air per total volume of bioreactor per minute) and 2% v/v (volume of CO_2 per volume of total aerated gas) [12]. 1-5% v/v CO_2 has been recommended generally to be mixed with air [14].

CO_2 as a source of carbon in addition to nitrogen determines the main type of productivity in algae culture. Although low C/N ratio, supports high biomass productivity, the high C/N ratio promotes higher lipid accumulation, hence for microalgae production for maximizing the biodiesel yield, the C/N ratio should be carefully manipulated [4]. As the atmospheric CO_2 is free and because of enormous carbon emission worldwide, some studies have considered using this resource for algae production [38]. Obviously increase concentration of CO_2 from stack gas can significantly increase the yield [39].

3.2.5. Nutrients

Nutrients play a very important role in photosynthesis and as follows lipid accumulation and biodiesel production. Proper concentrations of macronutrients including phosphorus and nitrogen are essential for lipid production [14]. Algae culture medium should be manipulated to maintain macro and micronutrients in appropriate proportions. Nitrogen and phosphorus are the main elements that should be considered [15]. Phosphorus is important for intercellular metabolism in form of polyphosphate molecules. In other words, high concentration of phosphorus should be available to promote luxury phosphorus uptake by algae (polyphosphate accumulation). Acid Soluble Polyphosphate (ASP) is crucial for metabolism and proteins and DNA molecules synthesis whereas Acid Insoluble Polyphosphate (AISP) is the storable phosphorus form which is capable to be used in external phosphorus shortages to handle the cellular growth demands [40].

Nitrogen is important in algae growth and lipid content accumulation and storage [32][2]. Nitrogen is assimilable for microalgae in form of Nitrogen-Nitrate (NO_3-N) and Nitrogen-Ammonium (NH_4-N) compounds. When both forms are available, microalgae prefer to take up ammonium but in ammonium deficiencies, nitrate assimilates to compromise the nitrogen demands for photosynthesis [14][21]. Furthermore, by altering nitrogen concentration levels we can accelerate or decelerate the lipids accumulation as the most important concept in lipid production and biodiesel extraction. In nitrogen repletion, intercellular lipid content is not especially high but the algae growth and reproduction advances. Once the algae experience nitrogen depletion, the grow rate decreases and lipid accumulation is the dominant activity associating with chlorophyll decrease [2][21][41][42][43]. Under nitrogen stress, lipid accumulations can reach up to 85% of algal dry mass in some cases by culturing appropriate strains (such as *Chlorella* sp.) [26][17][3]. It can be explained by nitrogen deficiency, we have lower synthesis rate of nitrogen into cell structural compounds like proteins and nucleic acids therefore lipid and hydrocarbon generation get higher rate due to carbon fixation[21][41].

Potassium (K) as one of the macro nutrients is necessary for photosynthesis and microalgae metabolism. Potassium is important element in enzymes structure, protein synthesis and osmotic control [44]. Wastewater and seawater contain high amount of potassium and magnesium which can fulfill microalgae growth demands. More than 0.5 kg potassium is needed to produce 1 kg of biodiesel. By recycling the water in the system and using sea or wastewater, amount of required external K can remarkably being reduced [23]. Some studies have done to use potassium as a catalyser for transesterification of microalgae oil to increase the biodiesel yield [45].

Microalgae biodiesel production consists of two main phases which are in compliance with retaining highest neutral lipid productivity. Microalgae should to be well treated to increase biomass as much as possible in the first step. With light, carbon dioxide, nutrients (nitrogen and phosphorus abundance) and other biological parameters, photosynthesis accelerates and microalgae can reproduce at a high rate and be prepared for the next phase. After biomass reached the optimum level, the lipid accumulation should be started. This normally happens after environmental stresses like nutrients or salt depletion [11][17][21]. In the second stage biomass production is replaced by lipid production. It has been shown that microalgae are capable to accumulate high amount of lipids under stresses which triggered from nitrogen depletion [4]. It has been exhibited that focusing on increasing biomass productivity is more important rather than lipid accumulation and storage to achieve viable biodiesel production [46].

Obviously carbon is essential for metabolism and lipid processing as 45% of microalgae composition is carbon [15]. The nitrogen level, together with carbon, controls either biomass production or the lipid yield in trade off reactions. Other minerals and trace elements and vitamins also are vital for cell metabolism and structural strength [4][1]. The optimum lipid production takes place when nitrogen starvation concomitants with other minerals availability [42].

As mentioned before, wastewater is nutrient rich resource for promoting microalgae production but to avoid side effects of wastewater use, it has been preferred to use artificial fertilizers to promote algae cultivation in researches and closed photobioreactors. Regardless to undesired aspects of using waste and seawater (such as biotic, toxic and unwanted solid organic matters), chemical fertilizers include high concentrations of nutrients in optimum ratios which support high algae growth [3]. Using artificial nutrient sources cannot be considered sustainable and viable solution for mass algae production because their production cost more than wastewater and their production process do not contribute in nutrients cycling.

Urine is enriched with nitrogen and phosphorus. In fresh urine, 85% of nitrogen is in form of urea and 5% in form of ammonia. The available phosphorus and nitrogen level and formation are variable by time. In (Table 2) urine composition is stated:

Table 2- Composition of urine. Derived from [33]

Parameter	Unit	Concentration	
		Household	Fresh Urine
Dilution	-	1.33	1
pH	-	9.1	6.2
N_{tot}	(g/m^3)	3631	8830
NH_4^++NH_3	(g/m^3)	3576	463
NO_3^-+NO_2^-	(g/m^3)	<0.1	-
P_{tot}	(g/m^3)	313	800-2000
COD	(g/m^3)		–
K	(g/m^3)	1000	2737
S	(g/m^3)	331	1315
Na	(g/m^3)	1210	3450
Cl	(g/m^3)	1768	4970
Ca	(g/m^3)	18	233
Mg	(g/m^3)	11.1	119
Mn	(g/m^3)	0.037	0.019
B	(g/m^3)		0.97

Chemical compositions of fresh and preserved urine are different. The fresh urine consists of higher amount of phosphorus-phosphate and less amount of ammonium-nitrogen than the stored urine. If urine is stored for 40 days, the ammonium-nitrogen concentration can reach to 3500 mg/L whereas the initial concentration is 190 mg NH_4-N/L. After this time, the ammonium level is steady to some extent. This transmutation time can be shortened by 25 days by adding urea to fresh urine. Urea hydrolysis to ammonia and bicarbonate raise the pH level from 6.5 to 8.8 during 10 days and 9.2 by approx 40 days storage. Free form of ammonia (NH_3) is volatile perilous component and can cause nitrogen loss and environmental problems [47][33]. Nitrogen in both forms of nitrate-N and ammonium-N is assimilable by algae cells although ammonium is preferable [21].

The reason for change of PO_4-P concentration is phosphorus precipitation via struvite ($MgNH_4PO_4.6H_2O$) crystallization, hydroxyapatite ($Ca(PO_4)_6(OH)_2$) and calcite ($CaCO_3$) generation [33]. This conversion accelerates with raising pH. Gethke *et al* has reported the PO_4-P level dropped from 164 mg/L to 110 mg/L within 30 day [47]. Adding metal ion-chelating agents EDTA (ethylenediaminetetraacetate) and sodium citrate can prevent phosphorus precipitation in long term and then phosphorus would be available if case urine should be stored for long time (e.g. for disinfection and hygienic reasons) [32].The positive side to feeding algae with fresh urine is lowering the pH by ammonia assimilation in addition to CO_2 bubbling. This process can help less phosphorus be precipitated before uptake by algae.

There are varying ways of feeding algae nutrients in photobioreactors. Mainly semi-continues feeding and batch feeding have shown good performance in algae grow. In batch feeding, nutrients is added to media at several separated times to support steady growth and maintaining the balance between nutrient uptake and nutrient availability but in semi-continues feeding, nutrients supply does not stop during the growth process term but the feeding rate is very low and based on hydraulic retention time, algae species, culture volume and etc. While Feng *et al* has recognized semi-continues feeding as the optimum solution, Schenk *et al* recommended batch feeding for enhancing the biodiesel yield [21][1].

Batch production follows the logistic curve based on the culture growth capacities like the volume of inoculum and the culturing conditions to promote algal growth [43][48]. Maximizing algal growth and reproduction can be subjected as the primary stage for biodiesel production. Supporting the exponential growth phase significantly depends on nutrients level. Phosphorus concentration is crucial parameter for promoting the microalgae metabolism and production including intercellular polyphosphate production. Hence, phosphorus level can be indexed as limiting factor for adjusting the nutrients abundant in the culture media in exponential growth phase. For example, if we assume that the phosphorus concentration in an urine sample is 500 mg/L and in the chemical fertilizer is 10g/L, for comparing urine and fertilizer in every volume of fertilizer has to be compared with 20 times volume of urine to provide the same concentration of phosphorus regardless of other nutrients concentration like nitrogen or potassium.

3.2.6. Light

Light is a basic requirement for photosynthesis. Light intensity, wavelength, timing and light attenuation induced by self shading are the main parameters which should be considered for optimizing microalgae yield. The optimum light intensity is the most important parameter should be manipulated to maximize the photosynthesis. Below the optimum point, light would be the limiting factor [12]. In the presence of nutrients, increasing light intensity provokes photosynthesis until the maximum algae growth reaches the light saturation point. After this point, increasing light intensity conduces to damages to light receptors of algae caused by photo-oxidation and decline in photosynthesis activities. The phenomenon is known as photoinhibition and can cause local heating and contamination of media with fast-growing microorganisms [2][15][14][12].

Furthermore Powell *et al* have demonstrated that raised light intensity has negative effect in luxury phosphorus uptake of algae. They have shown increase in light intensity does not influence acid-insoluble polyphosphate level but makes negative effect on acid-soluble polyphosphate generation which is important in intercellular metabolism and structure [32].

Increase in algae concentration can cause light penetration deficiencies in depths of culture medium due to absorption, light scattering and reflection [12]. At the closer side of reactor to the light source (illuminated side), cells obtain more light and block the light by their mutual shadows, hence the cells in dark side experience light shortages which so called cell-cell shading [37]. For example, in cell concentrations greater than 300gr TSS /m^3, light cannot attain more than 15cm of culture depth [2]. With aid of mixing, cells can reach same amount of incident light [1].

10-30 µmol photons/m^2 /s coupled with controlled temperatures has recognized as proper light intensity for stable microalgae cultivation [14] while some other studies have recognized higher ranges for light saturation point. Li *et al.* stated that intensity up to 600 µmol/m^2 /s was optimum level for algal starch and lipid generation for *Pseudochlorococcum* sp. [49] whereas Kumar *et al* had 30 to 45 W/m^2 (140-210 µmol/m^2/s) for the highest allowed light intensity for most of the algae species [12]. Light exposure time should be adjusted to obtain the best adaptation with algal natural metabolism and life cycle. 12/12 or 16/8 hours has recommended as light to dark hours ratio for most of microalgae species which matches daylight hours in many parts of the world [14].

Light source can be different according to the culture strategies and purposes. Generally, sunlight is the main source of light for outdoors cultivation and artificial light used for indoors microalgae production. The best light source for microalgae production is solar irradiance because it is a free sustainable light energy source which covers the full light spectrum, filterable for certain wavelength range to maintain the appropriate Photosynthetic Available Radiation (PAR) for the specific algae strain as target of algae culture. Variant duration of incident light of sun is the major problem of working with sunlight. Naturally in the absence of light, the algae switch their energy supply mode from autotrophic to heterotrophic [11]. Another point about using sunlight is controlling light intensity as sunshine can provides light magnitudes much higher than saturation point and cause photoinhibition. The light intensity of sunny days can reach 2000 $\mu mol/m^2/s$ [12] which is almost 10 times higher than advisable range.

3.2.7. Algae Strain and Cultivation Type

Although all the microalgae strains are able to reproduce and store intercellular lipids, only certain species are recommended for biodiesel production [42]. The ability of cells in rapid reproduction, high nutrient uptake, resistance to water and environmental deficiencies (e.g. temperature, pH, toxicity and etc), high lipid accumulation and high harvesting performance are determinant factors to choose an algae strain for biodiesel generation [2][14][11][17]. For example, *Chlorella* sp. is the most popular species which has shown the highest capability to increase lipid content up to 85% of algal dry mass [17][16].

It is important to determine algae metabolism reactions in different environmental conditions because algae can adapt its feeding system regarding to available carbon and energy sources and produce different levels of lipids. Accordingly, microalgae cultivation can be classified into 4 major algae feeding groups (metabolism) of Phototrophic, Heterotrophic, Mixotrophic and Photoheterotrophic. [11].

Phototropic algae use light and inorganic carbon (CO_2) as source of energy and carbon for photosynthesis (by chlorophyll) while some other species are also able to consume organic carbon as their carbon and energy source which called heterotrophic (nonphotosynthetic). Apparently, heterotrophic cultivation does not required light. [14]. The situation when microalgae are able to perform photosynthesis by absorbing both organic and inorganic carbon as source of carbon is mixotrophic. Actually, mixotrophic algae is biologically able to handle autotrophic and heterotrophic processes separated or simultaneously. The last group of cultivation is Photoheterotrophic which means algae needs light to apply organic carbon for its metabolism. Autotrophic is the major mutual algal metabolism for growth and reproduction but in this condition, the cell density and oil production are not considerable whereas heterotrophic growth can provide high lipid yield and more attractive for biodiesel generation [11][21][16]. Since one of the targets of biodiesel production is generating a carbon neutral fuel for reducing the GHGs emission, mixorophic algae cells are targeted for carbon sequestration (like *Chlorella zofingiensis*[16]). Because urine can assist them to reproduce in high levels based on autotrophic metabolism and then in absence of nutrient they can switch into heterotrophic mode to absorb and store organic carbons as source of inorganic carbon to stimulate higher lipid storage. It should be noticed that heterotrophic biomass production in absence of light, leads to more biomass and lipids production but the external carbon source like glucose or other organic acids makes it less feasible [50]. However screening the algae strains to determine the most efficient specie is important. Basically the lipids content of microalgae (% dry mass), lipid productivity (mg L^{-1} d^{-1}) and biomass productivity (g L^{-1} d^{-1}) are main criteria to choose the proper algae strain [22][51].

CHAPTER 4

MATERIALS AND METHODS

4.1. Overall Experiment Setup

To achieve the objectives of this study, experiments were conducted to identify microalgae *Scenedesmus quadricauda* responses to urine and phosphorus equivalent of other artificial fertilizer. Some primary experiments were done just to assess the basic behaviour of algae and this information have been used to conduct more advanced and target oriented experiments with replications.

Initially, the general information about the experiments and the experiments is described methodologies are explained later. The raw data have presented in appendices.

- The experiments took place at Kristianstad University between May-Sep 2011
- Air was injected into the culture [12] with the average rate of 0.5 vvm (volumes of air per total volume of bioreactor per minute).
- The circular agitation in the balloons was facilitated when the liquid level reaches the narrowed part of the balloon.
- Chlorophyll-a is considered as the algal growth indicator to measure photosynthesis activities and reactions performances [52][43] and, *Aqua*fluor™ (2004) by *Turner Designs Co*. was used to measure chlorophyll-a (Chl-a) level.
- pH and temperature values in culture medium were measured by pH meter Portamess® 912 (X) pH – manufactured by Knick Elektronische Meßgeräte
- The freshwater algae *Scenedesmus quadricauda* was used for the experiment.
- In all the measurements, phosphorous was measured as phosphate-P unless stated.
- The nutrients level was measured by standards methods:

Table 3- Chemical parameters and measurements standard methodology

Parameter	According to
Total-Nitrogen	Standard Methods 419A (Modified by Halina Rybczynsky)
Nitrate-Nitrogen	Standard Methods 419A
Total-Phosphorus	SIS 028127
Phosphate-Phosphorus	SIS 028126
Ammonia-Nitrogen	SIS 028134

Ten experiments were conducted which can be categorized into 2 parts; standardizing and growth experiments (Table 4).

Table 4- Experiments categories and titles

	Experiment	Experiment Title
Measurement Standardizing Experiments	Experiment 1	Fluorometer Application Range Examination and Reading Methodology
	Experiment 2	Fluorometer Calibration for Chlorophyll-a Value
	Experiment 3	Biomass Measurement
Microalgae Biomass Production Experiments	Experiment 4	Human Urine Competiveness with Artificial Fertilizer
	Experiment 5	Extended Urine Feeding
	Experiment 6	Algae Growth under High Urine Concentrations
	Experiment 7	Tap Water and Distilled Water-based Inoculums Cultivation
	Experiment 8	Aeration Timing Effects on Algal Growth
	Experiment 9	Comprehensive Comparison of Growth with Urine and Artificial Nutrient Sources
	Experiment 10	High Nutrients Availability Effects Assessment on Algae Growth

4.2. Chlorophyll-a and Dry Mass Measurement

In this section, series of the experiments have described which are used to collect, interpret and analyze data for other experiments. These experiments are designed for:

- Addressing the valid and trustable range of retuned values by fluorometer (Experiment 1)
- Assigning fluorometer return values to equivalent chlorophyll-a values in µg/L (Experiment 2)
- Measuring the dry mass in algae samples indirectly by spectrophotometery (Experiment 3)

4.2.1. Experiment 1 - Fluorometer Application Range

To determine chlorophyll-a level in culture, a fluorometer *Aqua*fluor™ (2004) by *Turner Designs Co.* was used to measure chlorophyll-a (Chl-a). The fluorometer is able to measure Chl-a with precision up to 0.1 ppb or 0.25 µg/L based on *In vivo* chlorophyll analysis technology [53]. Fluorometer measures the water (minimum 2ml) in the 10mm square non-glass cuvette and returns a number between 0-10000. Since light scattering and interference in high algae concentrations effect the return values, experiments were conducted to find out the reliability and calibration and determine the correct sample reading methodology.

Fluorometer produces numbers according to the chlorophyll content of water sample. The return value changes by Chl-a concentration in a linear trend until a certain level where the algae will interfere with the reading. To get to this level, concentrated algae sample was measured and diluted for several times until the displayed values became very low(<200). For every dilution, 3 readings were registered and the average of these readings considered as the dilution value.

The samples were taken in 0.2 ml volume and diluted in 16 ml or DI water (81 times dilution) to reach the below 1000 range values by fluorometer. Between 2 to 4 ml of diluted sample transferred to 10 mm cuvette to be inspected by device according to the manual. Obviously the actual value would be the returned values multiply a dilution ratio which is 81.

4.2.2. Experiment 2 -Fluorometer Calibration for Chlorophyll-a Value

To assign the fluorometer output to Chl-a level in standard unit (µg/L) series of water samples with different Chl-a values were used to compare the fluorometer output and spectrophotometer chlorophyll measurement [53][54]. Different concentrations of algae culture were prepared and the chlorophyll contents were examined with fluorometer. 200 ml of each culture filtered through GFC Whatman filter to measure Chl-a according to the Standard method SS 028 170. To compare the results of biomass in this study, returned values from Fluorometer are mostly presented. The term "Chl-a index" represents these values as indicators for biomass yield and consequently the trend of biomass levels.

4.2.3. Experiment 3 – Biomass Measurement

Algae dry mass can be used measure biomass changes [43]. Both direct and indirect methods can be applied. In direct method, certain amount of algae culture filtered through preburnt and preweighted Whatman GF-C, 47 mm glass-fibre filters. The digital balance used had a precision of 0.0001g. The filters after filtering were dried in a convection oven at 60°C for 18-24 hours. The initial weight subtracted from new weight (dry weight) gives dry mass. This weight should be divided by the volume of filtered culture to attain the dry mass of algae per volume of culture medium (g/L).

Wang *et al. (1979)* have stated that the algae concentration have direct relation with light absorbance. They suggested that 680nm wavelength can be used to measure the algal concentration in culture media because the most absorbance takes place at this wavelength [55]. Since there is a direct relation between biomass and dry mass and absorbance, we can measure the absorbance at 680nm to estimate the biomass. Seven samples of *Scenedesmus quadricauda* with different concentrations were examined in both absorbances by spectrophotometer and dry mass with the explained method. The filtered volume of algae was 250 ml. The filtered water was used to set the instrument to zero before measuring the absorbance. The regression between dry and absorbance was shown in a diagram.

Normally Chl-a level and dry mass have direct correlation. Therefore dry mass can be estimated by measuring the Chl-a level by Fluorometer. Different concentrations of *Scenedesmus quadricauda*were examined for Chl-a value and dry mass. The samples were taken from the stock container and diluted to cover a range of Chl-a indexes by Fluorometer. The samples were filtered and dried to measure dry mass. The graph of Chl-a and dry mass was generated by Microsoft Excel (Linear Regression).

4.3. Assessment of Culture under Human Urine

This experiment was designed to compare urine as a nutrient source for microalgae cultivation in compared to chemical fertilizer.

4.3.1. Experiment 4 - Human Urine Competiveness with Artificial Fertilizer

The goal of this experiment is to assess capability of human urine (HU) to promote algae growth in comparison with a commercial chemical fertilizer. For each nutrient source (urine and fertilizer) 6 samples of algae culture with different phosphorus concentration were prepared. Pittman *et al. (2011)* have shown that the algae growth rate is independent of starting cell concentration in wastewater, and different initial concentrations conclude to the same level of nutrient removal and algae growth [3] therefore our results are comparable regardless of initial algae concentration. Each sample consists of a glass balloon, contains 100 ml of algae from stock container which was mixed with 900 ml tap water and nutrients.

This experiment did not include any replication but was run as a series. The urine sample was fresh, therefore the ammonium concentration was relatively low. The main nitrogen compound in fresh urine is available in form of urea but with time, the urea degrades to ammonia and ammonium. Ammonium-N, urea-N and nitrate-N are assimilable by *Scenedesmus LX1* and can support the growth in the mentioned order [56]. The nutrient in the fresh urine sample were 53 mg nitrate-N/L, 410 mg P/L and 47 mg ammonium-N/L. 100 ml of the purchased fertilizer contains 5.1 g of Nitrogen (3.1g nitrate-N and 2.0g ammonium-N), 1.0g phosphorus, 4.3g potassium and approximately 11g of other minerals. Algal growth under phosphorus concentrations of 200, 500, 800, 1100, 1400 and 1700 µg P/L was examined. These phosphorus concentrations are equivalent to 0.48, 1.22, 1.95, 2.68, 3.41 and 4.14ml of urine and 0.02, 0.05, 0.08, 0.11, 0.14 and 0.17 ml of fertilizer. Fertilizer was diluted 10 times firstly and then 0.2, 0.5, 0.8, 1.1, 1.4 and 1.7ml of diluted solution of fertilizer added to containers. One container considered as control and did not receive any external nutrient (See Figure 3 as experiment arrangement map). This experiment was carried out in room temperature (19-22 °C).

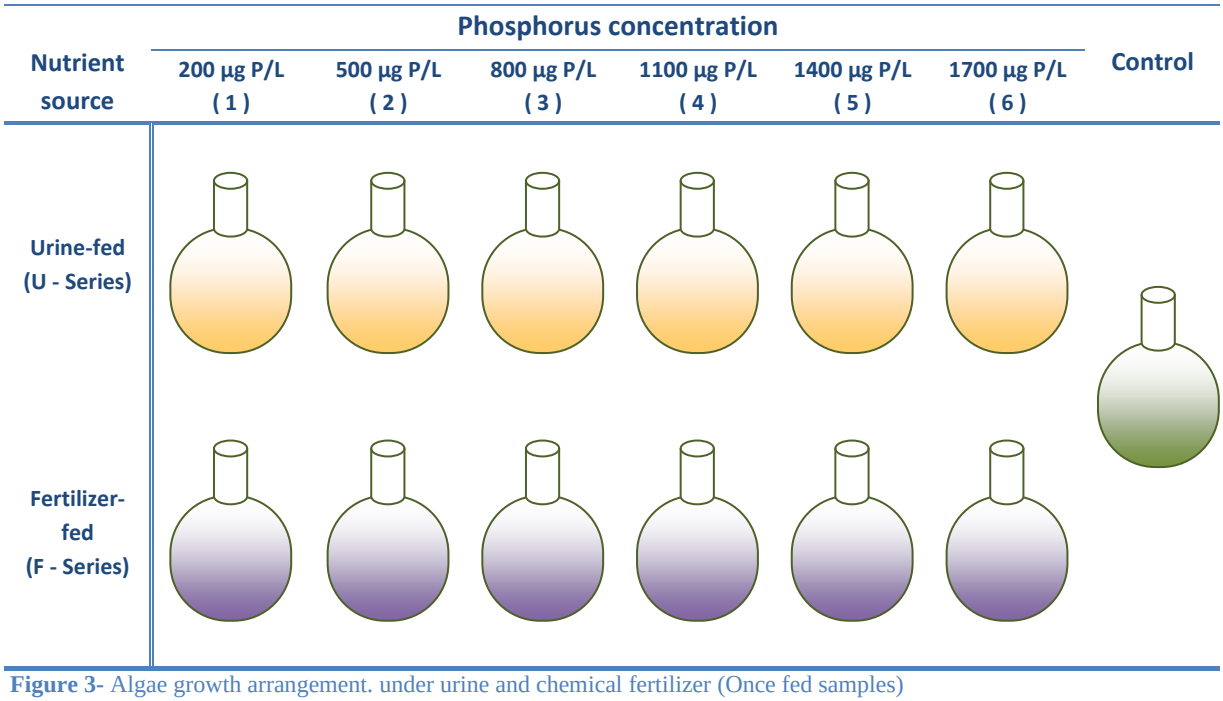

Figure 3- Algae growth arrangement. under urine and chemical fertilizer (Once fed samples)

Balloons were exposed to continuous air bubbling with a rate of 1000 ml/min and container were exposure to light intensity of 65 mmol m^{-2} s^{-1} provided by 4×36w Phillips fluorescent *Aquilights* for 12 hours a day from 06:00 to 18:00 (Figure 4).

Chlorophyll-a index has been checked between 17:00 to 19:00 p.m. every day. This initial test was carried out for 13 days then Chl-a decreased.

After 13 days, the culture colour became more transparent and yellowish with drop in chlorophyll content in all the vessels.

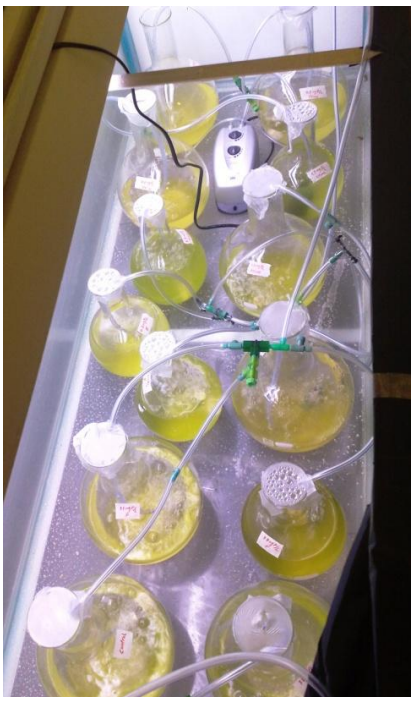

Figure 4- 13 glass balloon vessels used for urine-fertilizer comparison experiment.

4.3.2. Experiment 5 - Extended Urine Feeding

This experience was performed with urine series from Experiment 4, to understand the algae responses in periodical feeding (fed-batch) with equal phosphorus concentrations. This experiment was carried out in absence of fertilizer and control sample and the growth advancements were compared with earlier records of each balloon vessel separately.

The containers were fed 13, 19 and 26 days after the initial start of experiment 4. At day 13, 0.2; 0.22; 0.24; 0.26; 0.28 and 0.3 ml of urine (410 mg P/L) were added. Day 19 and 26, 0.3 ml was added to all containers. Daily measurements (between 19:00 to 21:00) were continued to 32nd day, after the decline appeared again in the general Chl-a level.

4.3.3. Experiment 6 - Algae Growth under Higher Urine Concentrations

In experiment 6 higher concentrations of phosphorus was used with fresh samples of algae. This experiment was conducted for 17 days and 6 different amounts of urine were used; 1.0, 1.4, 1.8, 2.0, 2.2 and 2.4 mg of phosphorus equivalent to 2.44, 3.41, 4.39, 4.88, 5.66 and 5.85 ml of urine (initial concentration of 410 mg P/L). The urine was added to glass balloon vessels containing 100 ml of algae from stock container and 900 ml of tap water. As Feng and Wu, 2011 suggested, the best result on *Spirulina platensis* biomass production takes place within 140~240 dilution ratios of urine [57]. The chosen volumes of urine, covered dilution rates of 171. These 6 samples were exposed to daylight with light intensity of 2500- 11500 lux (46 ~ 210 mmol m^{-2} s^{-1}) with daylight time of approx. 17 hours in June in southern Sweden. The experiment was conducted in room temperature between 20 to 23°C (see Figure 5 and 6).

Figure 5- Experiment conducted in 6 different concentrations and under indirect sunlight

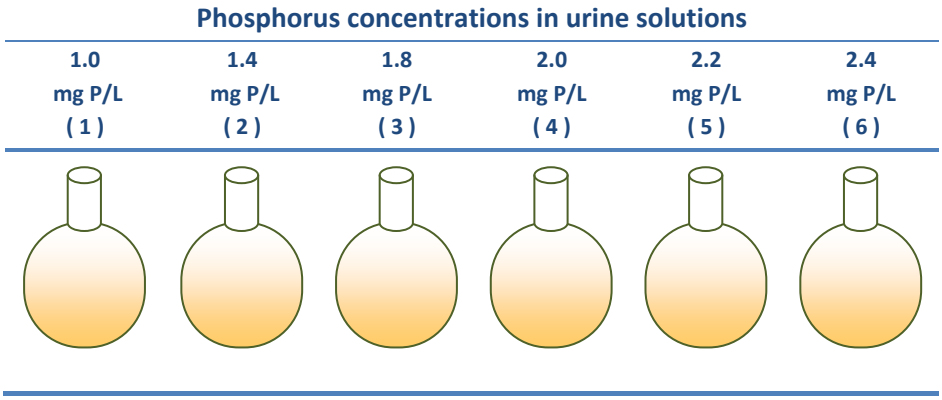

Figure 6- High phosphorus concentration experiment design plan

This experiment was designed to determine maximum possible chlorophyll concentration under high phosphorus concentration in single feeding system. The urine added to samples at day two to give algae time for adaptation to new culture. Since the growth model in this experiment did not follow the expected template, 10 days after urine addition, a second urine feeding was done. All the containers received 1.8 mg phosphorus (average phosphorus quantity in the first round). Continuous air bubbling with rate of 1 lit/min was applied for each container. Sampling and measurements ran everyday between 19:30 – 23:00 to determine daily synthesis results. In this experiment, the algae concentration reached the highest values in all of the conducted experiments, requiring dilution ratios up to 160 times for the fluorometer.

4.3.4. Experiment 7 -Tap Water and Distilled Water-based Culture

Water chemistry is important for alga growth since it influence the ionic and pH equilibrium of intercellular and cellular constituents and influence nutrients uptake and photosynthesis. In this experiment, tap water (TW) and distilled water (DW) was compared by monitoring the culture growth with the same biological and environmental parameters such as nutrients level, aeration, temperature light and etc. Prior to the main phase of this experiment, a simple experiment done to gain the main idea of possible differences in the urine growth capacities in distilled water (DW) and tap water (TW). This experiment was done without replication and shown considerable differences in chlorophyll contents hence a comprehensive experiment was conducted with 3 replications. Every series, consist of 1.9 lit water with different sources (either TW or DW) in addition to 100 ml algae culture from stock container. Containers were located behind the window reaching daylight sunlight in July in southern Sweden (Figures 7 and 8). The containers were aerated with rate of 0.8 lit/min (0.4 vvm) and temperature range was 21-25°C.

Four ml of fresh urine (450 mg P/L, 68 mg NO_3-N/L and 46 mg NH_4-N/L) was added to each container. Full-time aeration applied and data collection took place every day between 20:00 to 22:00. pH was measured every second day. This experiment was carried out for 10 days.

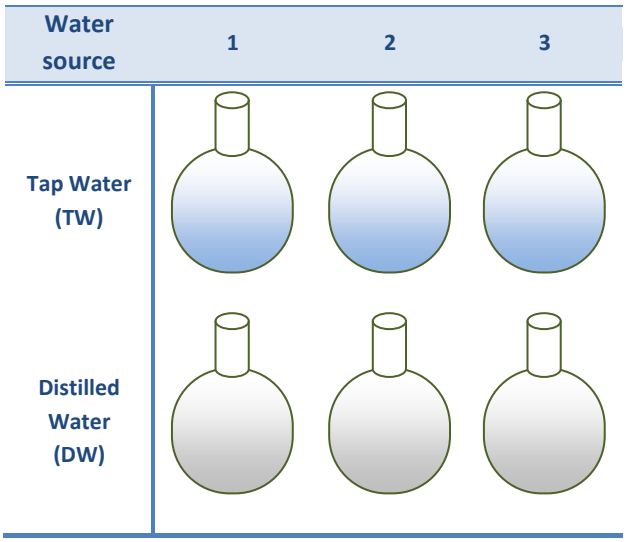

Figure 7- Distilled Water (DW) vs. Tap Water (TW) design plan

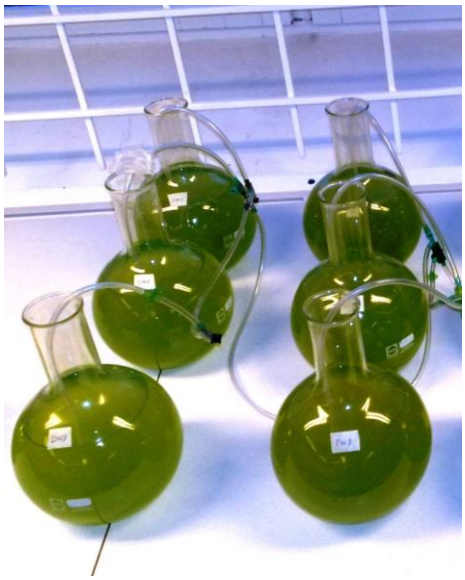

Figure 8- Distilled water (DW) comparing to tap water (TW) growth in 3 replicas

4.3.5. Experiment 8 – Aeration Timing Effects on Algal Growth

This experiment was carried out to assess how timing in air bubbling can affect algal growth. For this purpose, 3 series of samples, each includes 3 replicates were used, No Bubbling (NB), Half-time Bubbling (HB) and Full-time Bubbling (FB). In NB series, the containers did not receive any aeration and stirring within the 14 days of experiment. In series with half-time bubbling, 16 hours bubbling took place and 8 hours (between 21:00 to 05:00) no aeration was done. In FB series, every vessel received 24 hours aeration. Aeration intensity for the HB and FB samples was adjusted to 0.4 vvm (volume of air per volume of culture per minute) evenly.

The experiment begun in early July 2011 and samples were illuminated by natural light behind window in 22-25 °C (Figures 9 and 10). Every sample consists of 100 ml algae from stock container in addition to 2L of tap water and 4ml of urine with 445 mg P/L value. Data collection carried out between 21:00 to 23:00 every evening using Aquafluor. The light intensities fluctuated from 7 to 12th days of experiment due to intermittent changes between sunny and cloudy days.

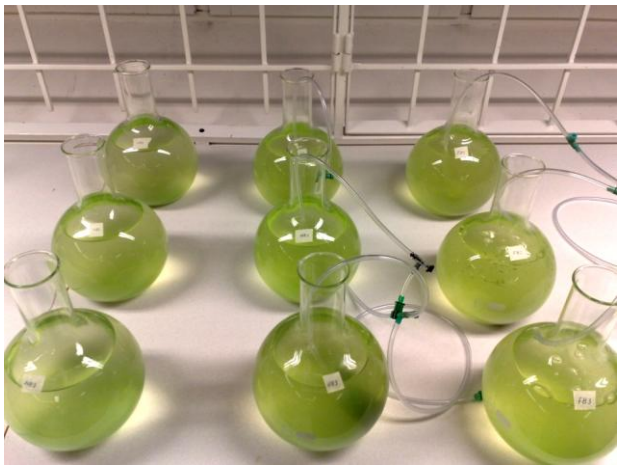

Figure 9- Left to right: No Bubbling(NB), Half-time Bubbling(HB) and Full-time Bubbling(FB) series

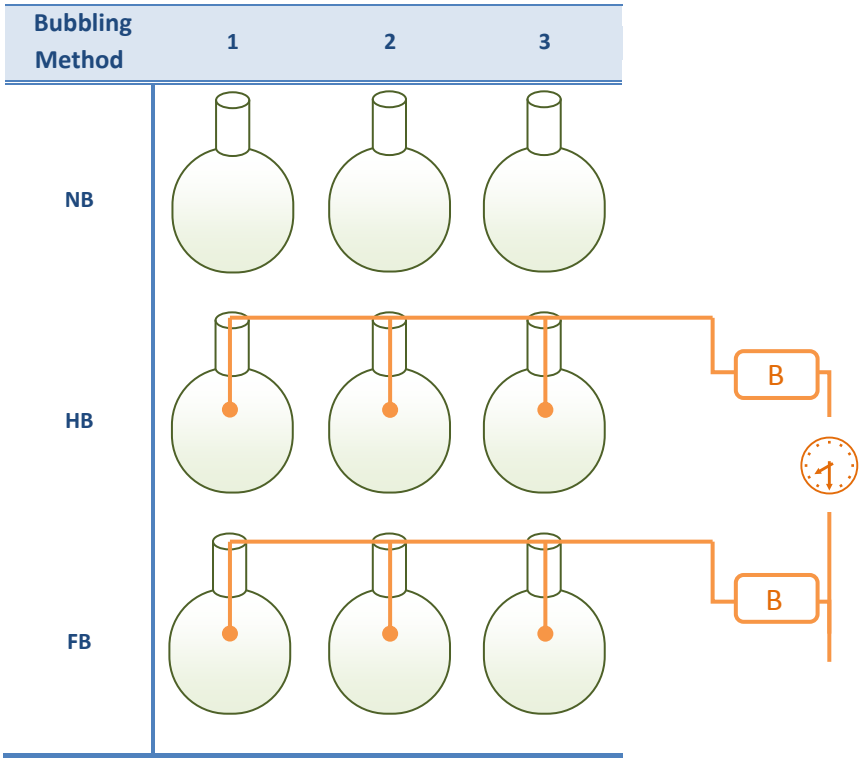

Figure 10- Bubbling experiment arrangement plan. Bubbler (B) in HB series was connected to timer for 16h bubbling a day

4.3.6. Experiment 9 –Comparison of Growth with Urine and Artificial Nutrient Sources

This experiment can is the main experiment within this study. Experiment 9 was conducted to apply the results from previous experiences and to measure the growth under more controlled conditions. This experiment was conducted to compare Phosphorus-equal (520 µg P/L) cultures. The nutrients sources were urine, Bold's Solution and artificial fertilizer used in experiment 4. An extra series with higher phosphorus concentration (1800 µg P/L) made to compare higher concentration of urine with lower one. 2L glass balloon vessels were used with 5 replicas which were placed in a photobioreactor (light-sealed rectangular glass container) to prevent external light reach the samples. The algae from stock container (Figure 11) used (50 ml of stock per 2250 ml of medium).

Figure 11- *Scenedesmus quadricauda* Algae stock culture, containing 0.32 g/L dry mass

Aeration with rate of 0.9 l/min (0.4 vvm) was taking place for 16 hours a day from 06:00 am to 22:00. Illumination was performed 16 hours a day as the same time as aeration. Lighting was provided by 4 Philips fluorescent lamps. Including pair of 36W daylight and pair of 36W *AQUA RELLE*, providing 3500 lux (65 µmol m^{-2} s^{-1}) light intensities which was monitored at the surface of the reactors using a PAR (photosynthetically active radiation) irradiance sensor. Temperature was 30°C during illumination and 28 °C in the dark. The macronutrients level in urine was 190mg PO_4-P/L, 46 mg NH_4-N and 142 mg NO_3-N/L. In many literatures, Bold's solution [58] has considered as a well formulated artificial algae culture [17][59][60]. The Bold's formula was modified to provide higher nitrogen values to support more efficient exponential algal growth. The Bold solution consists of 10 stock solutions mentioned in Table 5. Phosphorus and nitrogen in stock solution are 53.25 mg P/L.

Table 5- Bold's modified formula

Stock	Solutions	Stock Concentration	Unit	Concentration in 1L culture medium (ml/L)	Concentration in 1L of Culture Medium (mg)
Stock 1	KH_2PO_4	17500	mg/L	10	175
Stock 2	$CaCl_2 . 2H_2O$	2500	mg/L	10	25
Stock 3	$MgSO_4 . 7H_2O$	7500	mg/L	10	75
Stock 4	$NaNO_3$	75000	mg/L	10	750
Stock 5	K_2HPO_4	7500	mg/L	10	75
Stock 6	NaCl	2500	mg/L	10	25
Stock 7	H_3BO_3	11420	mg/L	1	11.42
Stock 8	Na_2EDTA	10000	mg/L	1	10
	KOH	6200	mg/L		6.2
Stock 9	$FeSO_4 . 7H_2O$	4980	mg/L	1	4.98
	H_2SO_4	1	ml/L		0.001 ml/L
Stock 10	$MnCl_2 .2H_2O$	1440	mg/L	1	1.44
	$ZnSO_4.7H_2O$	8820	mg/L		8.82
	$NaMoO_4 .2H_2O$	1079	mg/L		1.079
	$CuSO_4 .5H_2O$	1570	mg/L		1.57
	$Co(NO_3)_2 .6H_2O$	490	mg/L		0.49

The urine and fertilizer quantity were adjusted to provide the same level of phosphorus in culture as Bold solution (0.53 mg P/L). Initially, 11.5 L of media was made and then divided into vessels by 2250 ml for each. The rest were filtered and preserved for chemical measurements. 2.79 ml of urine and 0.053ml of fertilizer provide 0.53 mg P/L in culture; therefore, 32.1 ml urine and 0.61 mg fertilizer were dissolved in 11.5 lit of media.

To investigate the productivity in higher concentrations of urine, a series of inoculums with 1.80 mg P/L was arranged. To supply this level of phosphorus 109 ml of urine was needed for 11.5 L of culture medium. The experiment arrangement plan and photos are presented in Figures 12 and 13.

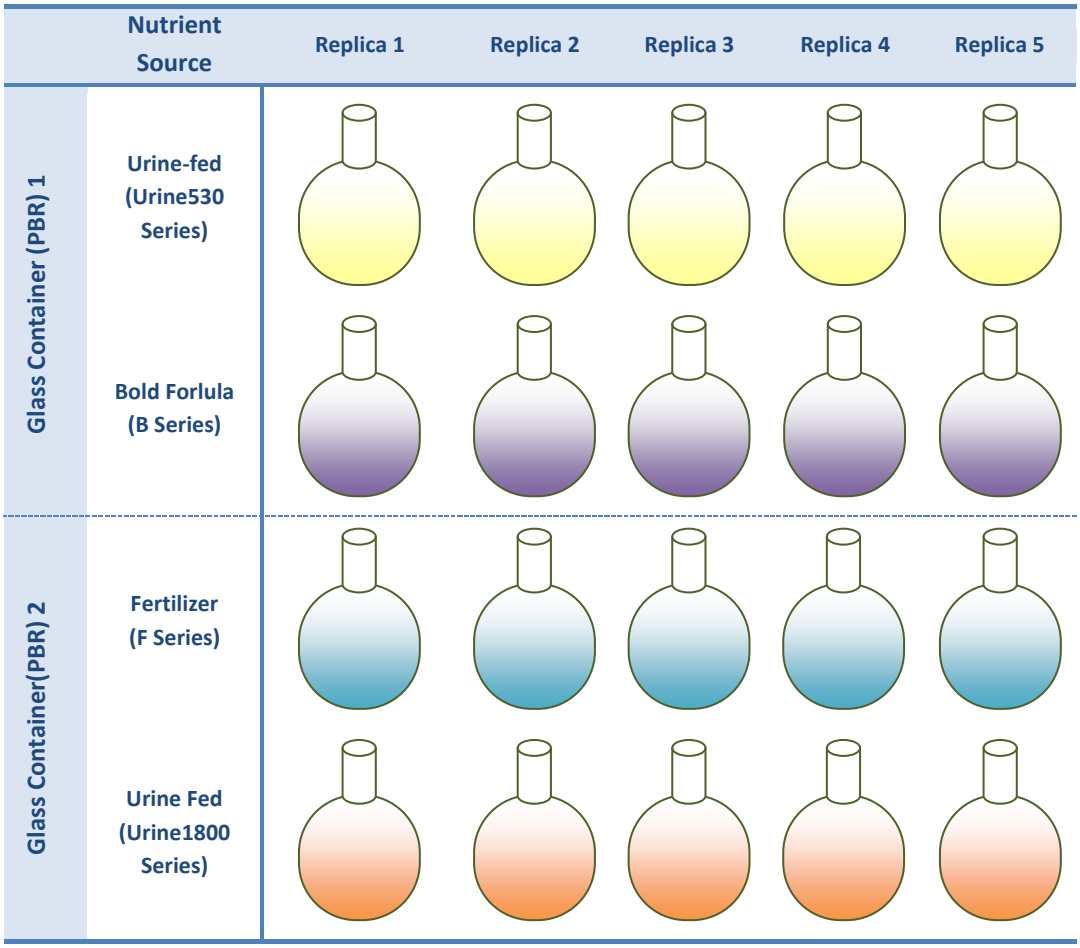

Figure 12- Experiment 9 arrangement plan

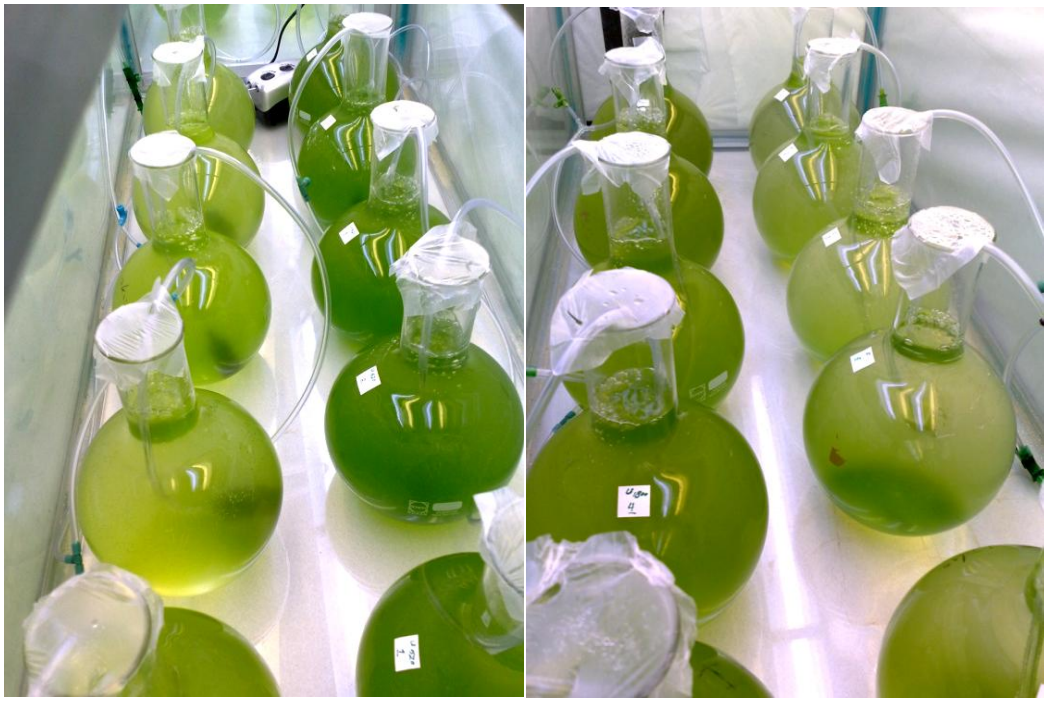

Figure 13- Left to right: Bold and Urine 530 series (Container 1), Urine 1800 and Fertilizer series (Container 2). Day 3 after experiment setup

Since in previous experiment the growth peak had taken place after 5 days of first feeding, it was decided that re-feeding with the initial amount of nutrient performs at 5^{th} day after experiment setup was to be done. This is to support continuous algal production by feeding at peak points when the nutrients in media are in minimum due to uptake by algae. On day 5, 100 ml of culture was filtered and replaced with the respective initial concentration in each series. The filtered culture samples were preserved in -18°C for nutrients measurement. 0.5 ml of H_2SO_4 was added to 50 ml of filtered water for fixing the phosphate-P level. Initial dry mass measured using Equation 4 on the first day and repeated for urine series on day 4 and day 9 because during the experiment low or negative biomass production occurred in Bold and Fertilizer series. This experiment was conducted for 7 days, until the chlorophyll level in fertilizer and Bold series shown a permanent decline but continued for till day 9 for urine series considering their positive growth. Urine series received additional urine on day 7 because urine series were demonstrating an overall positive growing trend. Then 200 ml of culture in every replica was replaced with 200 ml of solution. Data collection including the Chl-a level and pH measured every day between 20:00 to 22:00. Standard deviation and average of Chl-a index in pH were calculated by Microsoft Excel.

4.3.7. Experiment 10 – Assessment of High Urine-Nutrients Effects on Algae Growth

This experiment was conducted to determine the highest possible yields dependent on phosphorus concentration. The effects of high values of phosphorus concentration was investigated in experiment 6 but the limited range, no replication are the main reasons that the results from experiment 6 should be revaluated in more standardized manner. The phosphorus concentration in this experiment was 2.0; 2.5; 3.0 and 3.5 mg P/L with 5 replicas.

20 of 2L glass balloons were filled by culture medium, 2100 ml of tap water plus 50 ml of stock culture (with Chl-a index of 82500). The urine sample was collected 3 months before the experiment runs, therefore the ammonia-N level was relatively high (2888 mg NH_4-N/L compared to fresh urine with 344 mg NH_4-N/L). Total Nitrogen in the urine sample was 6986 mg N/L and total Phosphorus was 421 mg P/L. The nitrate level measured as 84 mg NO_3-N/L. 10.25, 12.8, 15.36 and 17.92 ml of urine were added to supply 2.0, 2.5, 3.0 and 3.5 mg P/L in 2 series respectively. Chlorophyll-a was measured with fluorometer (everyday at 9:00 p.m) and indirect dry mass measurement with spectrophotometry method (Experiment 3) every 3 days. The aeration and illumination were part time (16 hrs lighting and bubbling over 8 hrs darkness without bubbling). Two boxes each contain 2 series of sample were equipped with pair of Phillips TLD 36W fluorescent lamps which provide 1750 lux (32.2 mmol m^{-2} s^{-1})(Fig. 14) bubbling rate was of 0.8L vvm and average temperature 26°C.

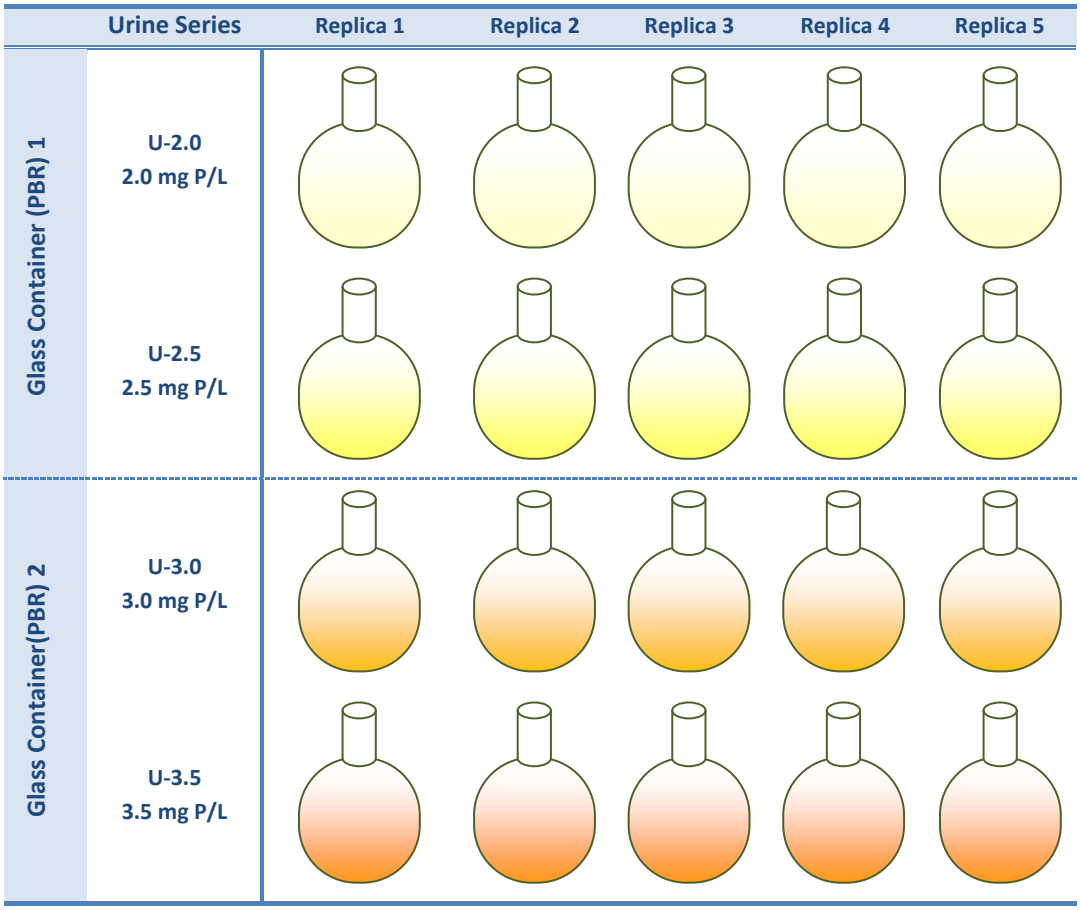

Figure 14- Experiment 10 arrangement plan

At day 5 of experiment, the same amount of urine was added to promote steady growth. Next round of feeding was carried out on day 8 and 11(Table 6).

Table 6- Feeding proportions and schedule in Experiment 10

Urine Series	Feeding at day 1 from 421 mg P/L urine	Feeding at day 5 from 421mg P/L urine	Feeding at day 8 from 385mg P/L urine	Feeding at day 11 from 385mg P/L urine
U-2.0	10.25ml	10.25ml	5.0ml	6.17ml
U-2.5	12.8ml	12.8 ml	6.0 ml 150 ml culture replaced	7.96 ml
U-3.0	15.36ml	15.36 ml	7.0ml 150 ml culture replaced	9.75ml
U-3.5	17.92ml	17.92ml	200ml of culture replaced with tap water(Exceptionally)	-

For the next round, urine with 385mg P/L and 342 mg NH_4-N/L values were utilized. At day 8, the urine feeding procedure, consist of replacing 150ml of culture with 150ml of urine solution with the respected concentrations (Table 6) and also to support the nutrients in inoculums with minerals available in fresh tap water. In U-3.5 series after observing continuous negative response in algae production after day 5, it was decided to replace the 200 ml of culture with 200 ml tap water to see if dilution can influence the growth after a shock of a high nutrients level. The experiment was shut

down for U-3.5 after 10 days and after 11 days for U-3.0 series. The experiment continued till day 17, for U-2.0 and U-2.5.

CHAPTER 5

RESULTS AND DISCUSSION

In this chapter, results of experiments has been presented and discussed in the order of the experiments described in previous chapter. In all the graphs, standard deviation is presented as error bars.

5.1. Experiment 1 - Fluorometer Application Range Examination and Reading Methodology

Figures 15 and 16 illustrate the relation between returned values of fluorometer and dilution times.

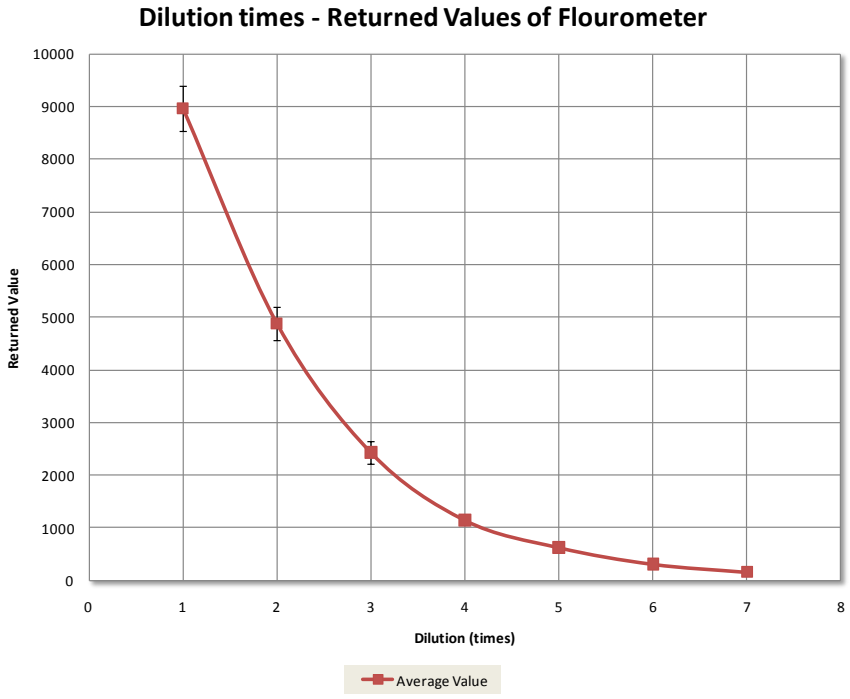

Figure 15- Fluorometer returned values upon increase in culture dilution rate.By increasing the dilution, we get closer to linear values

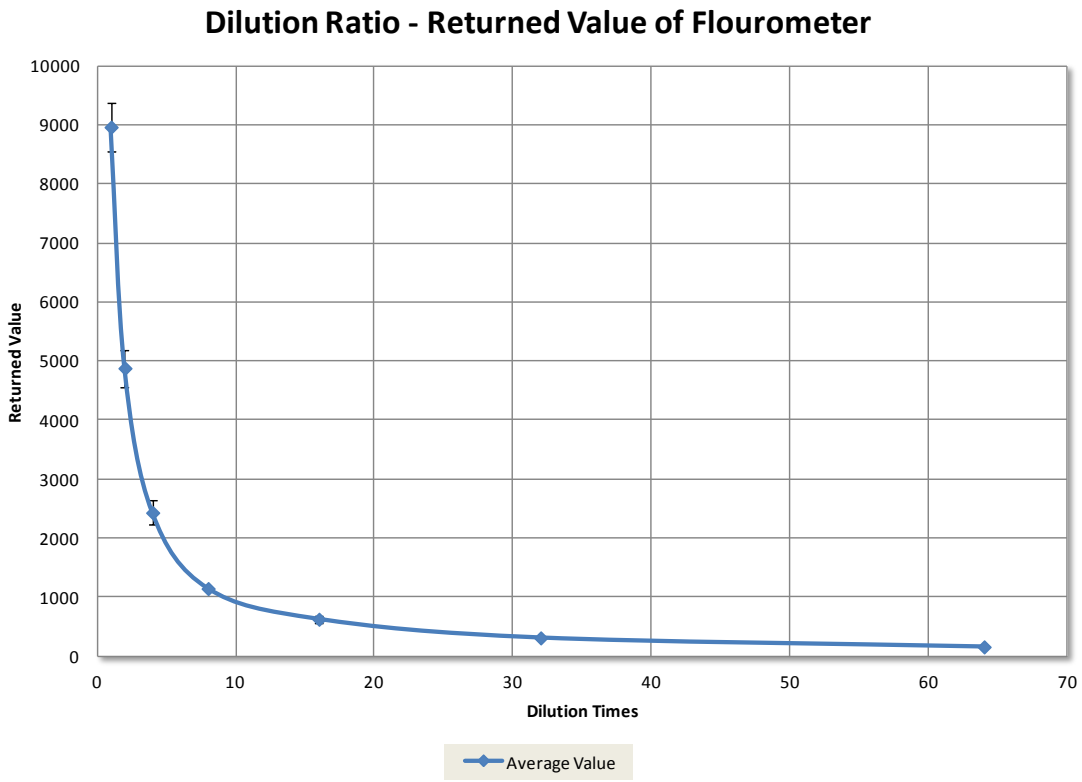

Figure 16- Dilution ratio – Returned values chart magnifies the linear part of graph (below 1000). The standard deviations are not considerable in linear range

Results of this experiment were used for other measurements. As shown, the linear section of the chart showed up in the range below 1000 therefore in our measurements dilution was done to reach the value less than 1000. Standard deviation of values of triple measurements in this range showed a dramatic decreased which shows a good reading precision in low concentrations of chlorophyll-a.

5.2. Experiment 2 -Fluorometer Calibration for Chlorophyll-a Value

Results of fluorometer calibration experiments are shown in Figure 17. This chart provides formula to turn fluorometer return values into chlorophyll-a concentration. The linear correlation gives a reliable equation (R^2= 0.9597) for further calculations (Eq. 3).

$$\text{Chl-a}_{C} = 0.0926 \times V_F + 4.324 \qquad (3)$$

Where V_F is the value which is returned by fluorometer and Chl-a_C is chlorophyll concentration in µg/L.

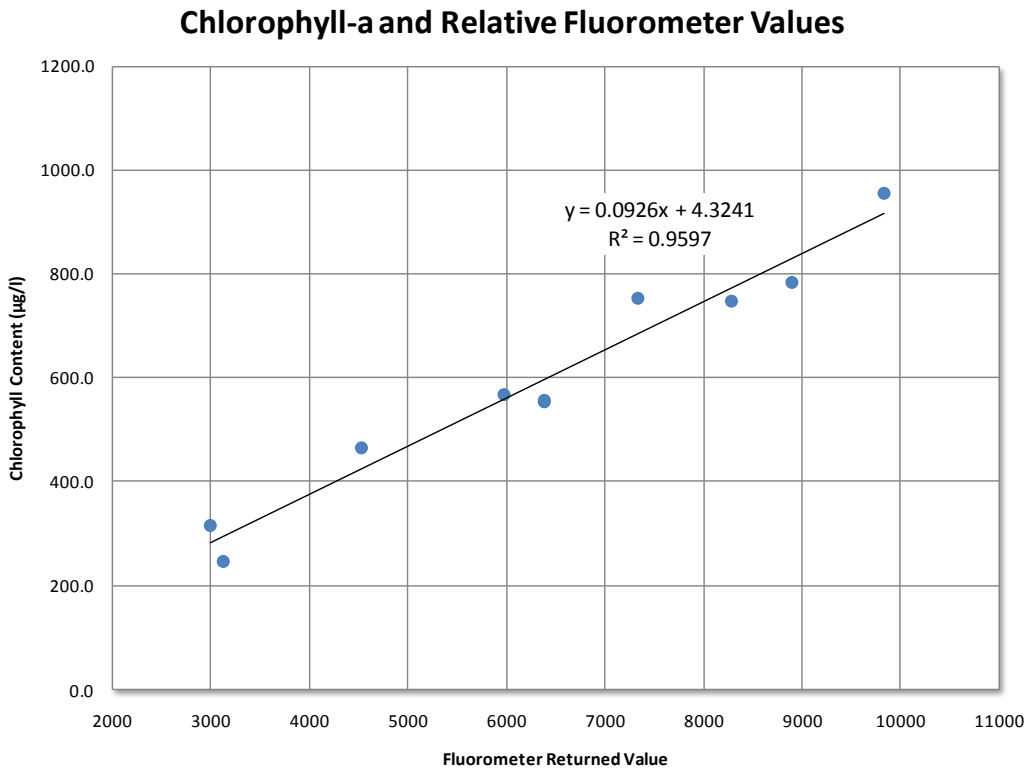

Figure 17- Relative chlorophyll-a content of fluorometer readings and respective equation

5.3. Experiment 3 – Dry Mass Measurement

For *Scenedesmus quadricauda*, dry mass and light absorbance at 680nm shows a linear relationship (Figure 18). We are able to get an estimate of the dry mass very quickly and precisely without taking out any proportion of culture medium. Equation 4 is applicable for *Scenedesmus quadricauda* according to special cells shapes, size and content.

$M_d = 0.02372\ Abs_{680} + 0.019 \quad (R^2 = 0.9928) \qquad (4)$

Where M_d is dry mass in g/L and Abs_{680} is absorbance of *Scenedesmus quadricauda*culture at 680nm wavelength. This linear relation is applicable for the algae concentration up to 200 mg/L.

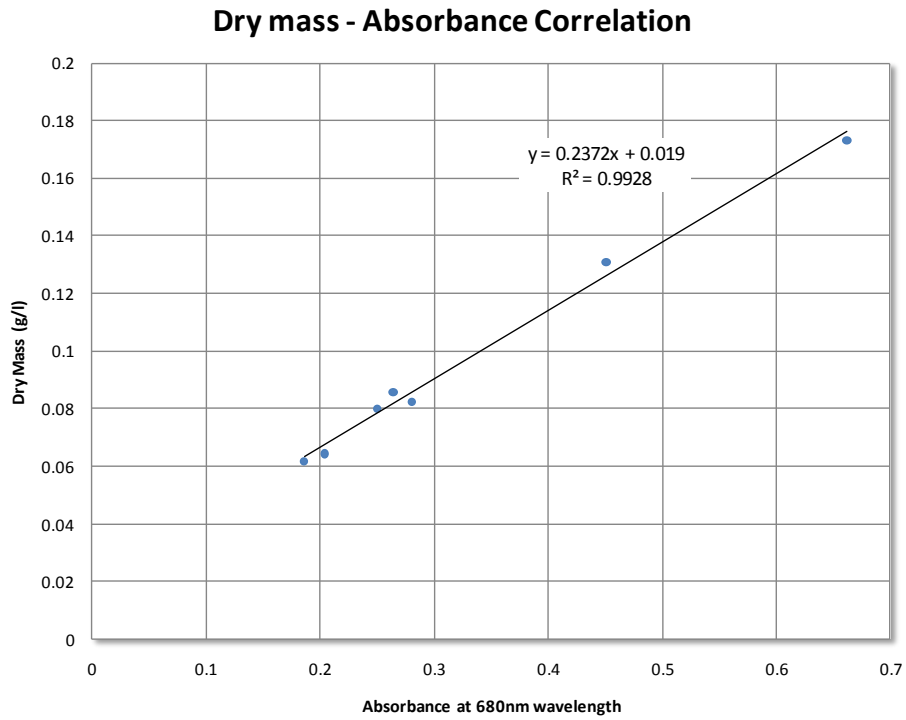

Figure 18- Absorbance - Dry mass relation on *Scenedesmus quadricauda*

To assess the dry mass by the fluorometer, Chl-a indexes and their relative dry mass measurement (direct dry mass measurement) is presented in figure 19. The range of the Chl-a indexes covered a range of 3402-54675 and their dry mass range varied between 10.9 to 110.8 mg/L.

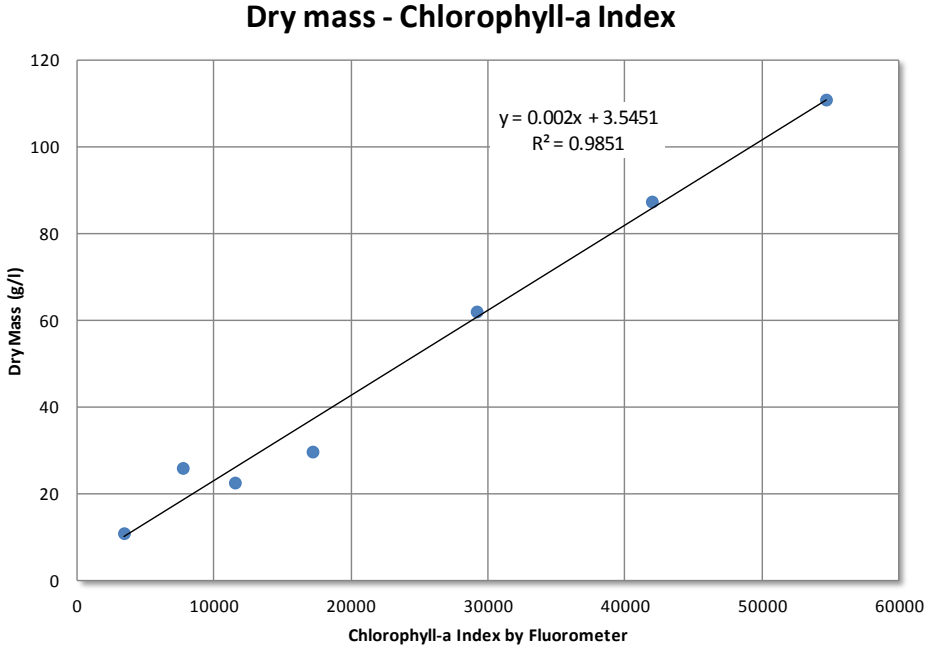

Figure 19- Correlation between dry mass and Chlorophyll-a index by Fluorometer

Equation (5), can be used for Chl-a index to dry mass conversion

$M_d = 0.002\ I_{Chl\text{-}a} + 3.5451 \qquad (R^2 = 0.9851) \qquad (5)$

Where M_d is dry weight in mg/L and $I_{Chl\text{-}a}$ is chlorophyll-a index by fluorometer.

5.4. Experiment 4 and Experiment 5 - Human Urine Competiveness with Chemical Fertilizer and Batch Feeding Assessment

The results of urine-fed and fertilizer-fed algae can be seen in Figures 20 and 21. In both series, a "standard" growth curve is observed. The first growth phase extends up to 5 days and then a senescence phase is distinguishable. The maximum value for urine fed algae was more than 40% higher than fertilizer-fed algae (Fig 20, 21).

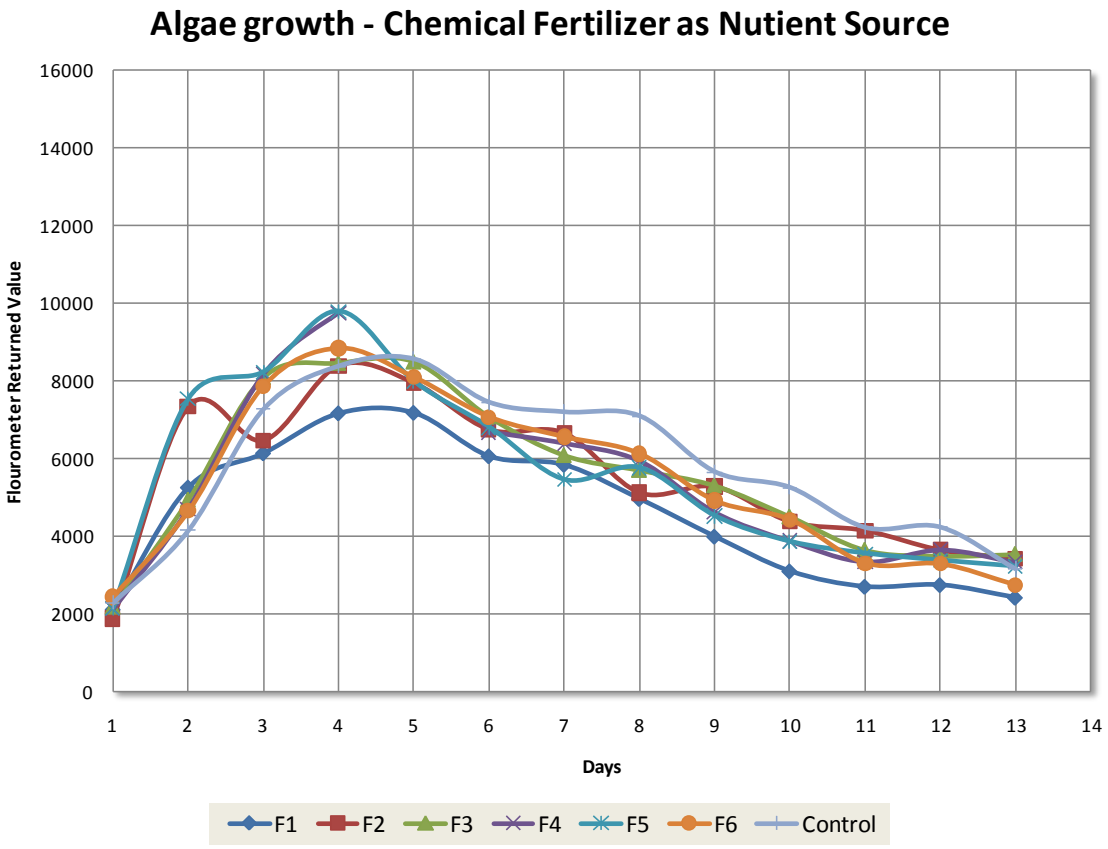

Figure 20- Fluorometer results of fertilizer-fed algae growth in experiment 4. F1, F2, F3, F4, F5 and F6 are representing phosphate-P concentrations of 200, 500, 800, 1100, 1400 and 1700 µg phosphate-P/L respectively.

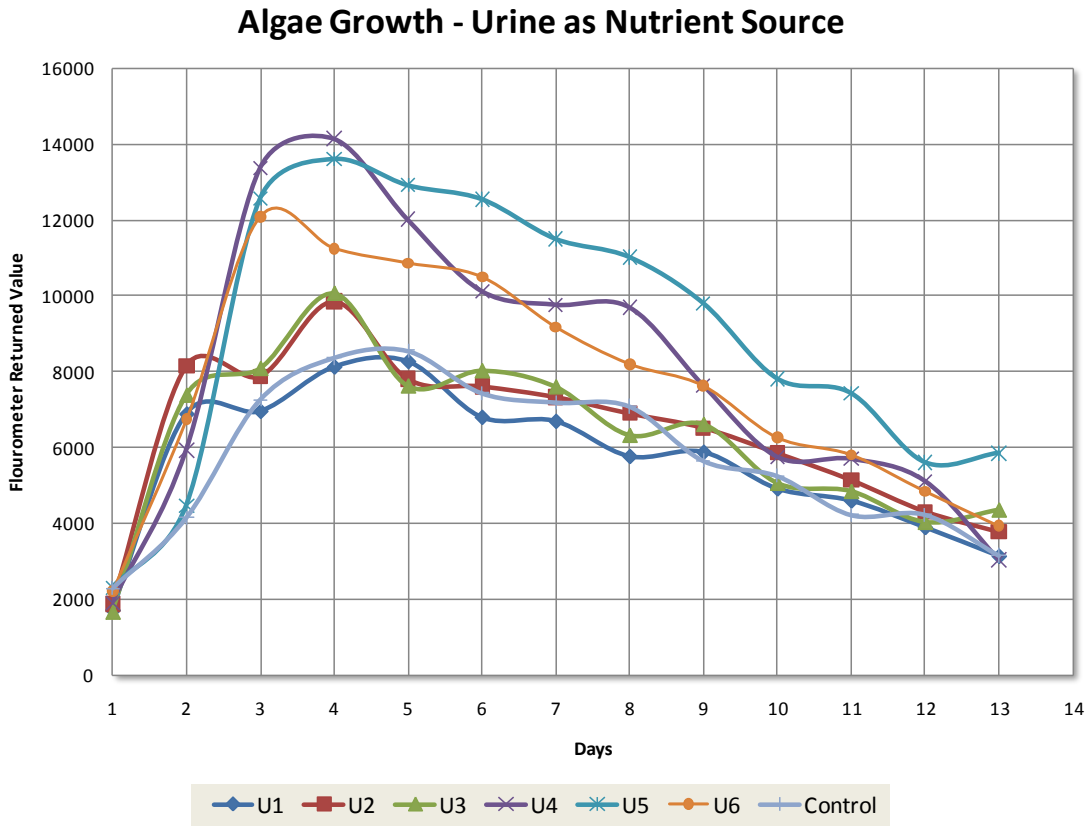

Figure 21- Fluorometer results of urine-fed algae growth in experiment 4. U1, U2, U3, U4, U5 and U6 are representing phosphate-P concentrations of 200, 500, 800, 1100, 1400 and 1700 µg phsphate /L respectively.

The control gives the baseline growth curve. The chlorophyll-a levels above the baseline can be considered as function of nutrients availability of external sources (either urine or chemical fertilizer). Containers with Phosphorus levels of 1100 and 1400 µg/L showed the highest chlorophyll production as indicator of algae growth in both nutrient series. Urine fed inoculums with phosphorus values of 1100, 1400 and 1700 µg P/L, were performed promising algal growth reaching biomass values as high as 32.37 mg/l in U4 vs. 23.13 mg/l in F5.

After the 5th day, an inclusive decline in Chl-a content was observed in all the samples. This phenomenon can be interpreted as nutrients shortage in the medium after reaching the highest productivity before the maximum point. We can assume that the culture have entered the declining growth rate phase. In nutrients abundance, phosphorus and nitrogen have contributed in cellular membrane and biomass generation which is supported by photosynthesis activities. This phase consumes phosphorus and nitrogen by means of nutrients assimilation by microalgae [21][43]. When the nutrient availability is not enough to promote more chlorophyll synthesis, cellular metabolism switches to heterotrophic mode. This process is associated with intercellular protein content decline due to chlorophyll degradation and sugar and lipid accumulation [49][42]. This explanation is compatible with chlorophyll level decline. It is expected to attain higher lipid storage in this phase [21][41][42]. Using in-vivo chlorophyll analysis is not suggested to measure the biomass and lipids storage in stationary stage because the decline in pigments does not mean the decline in cells and metabolism activities [43]. The reduction in Chl-a indexes continued in all samples. After 13 days the Chl-a levels in all the samples was relatively close.

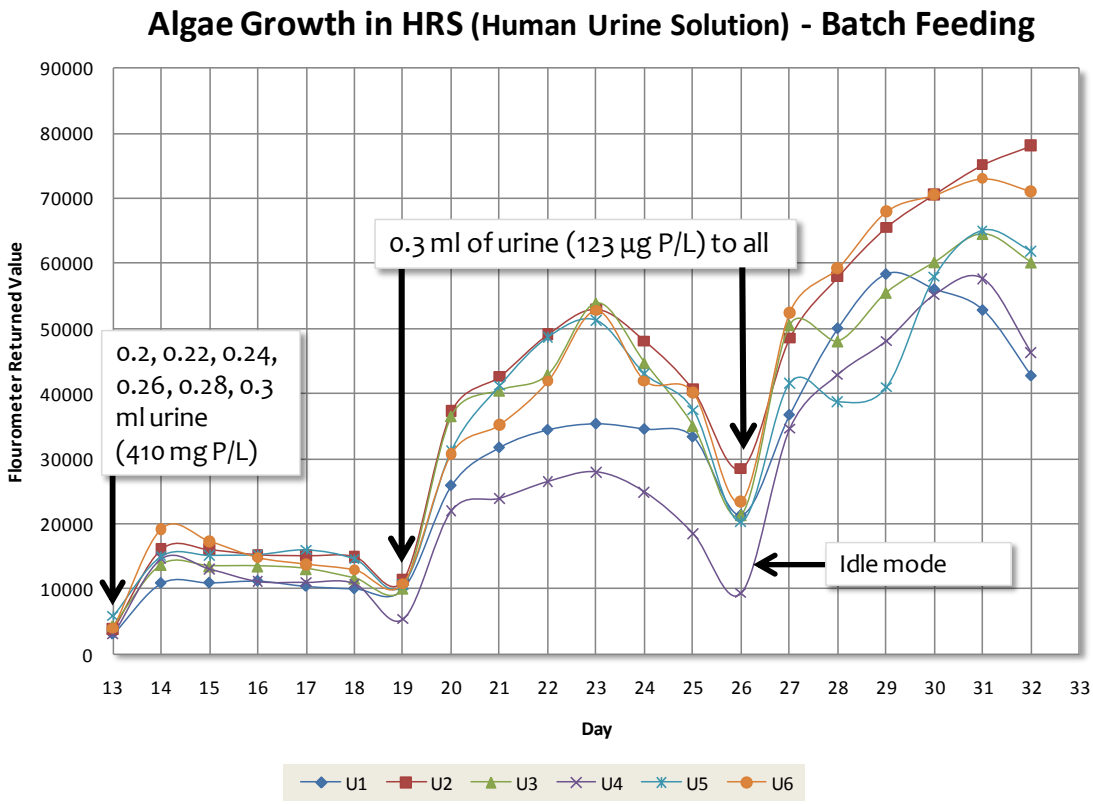

Figure 22 - Periodical growth supported by periodical HU feeding after the primary growing experiment (Experiment 4). At day 13, 0.2, 0.22, 0.24, 0.26, 0.28, 0.3 ml of urine with 410 mg P/l injected into U1-U6 container. 0.3 ml of urine (123 µg P/L) added on days 19 and 26 to all containers.

Low quantities of urine was added at 13, 19 and 26th day of Experiment 4 (Fig. 22). The sharp growth recovery was observed immediately after the samples received nutrients. This growth was maximised in U6 sample (with 0.3 ml urine receive) by rate of 490% between day 13 and day 14. The results after one day showed nutrients shortage and the high nutrient uptake capacity after a starvation. The important point about this feeding stage is that the Chl-a level reached a higher level than during first 13 days, although the received nutrient levels were noticeably lower (range of 80-120 compared to 200-1700 µg P/L). This indicates the first main feeding phase produced intracellular membranes and in the next low level nutrients availability, the photosynthesis organs and chlorophyll generation was mainly supported in existing cells [49] otherwise the nutrients would be consumed for supporting production of cellular constituent elements and thus, Chl-a level could not reach higher level than the prior phase. As demonstrated in the graph, the nutrient at this level was not enough to support growth more than one day and the slight decrease continued. Six days after first feeding in the second phase of experiment (day 19 after start date) an unexpected drop appeared in all samples. U4 experienced bigger decline and than other samples which influenced its behaviour the rest of the experiment. Algae in U4 started to flocculate and sink to the bottom. When algae entered to this condition, its responses to nutrient repletion was became slower and lower than normal conditions. This reaction observed in other experiments as well and it causes diminishing in photosynthesis process and Chl-a generation. In U4 case, this sample had followed the general course but after the 19th day its chlorophyll generation capacity got lower. U4 reaction is algae unrepresentative and should be excluded. This condition happened for some other samples during our experiments which are referenced to as ‘idle mode’.

At 19th day of the experiment, the nutrient depletion put all the samples in similar Chl-a level (except U4). 0.12 mg P was added to all the samples and we can see the fast growth rate up to 360% within 1 day. The increase continues for 4 days with lower rates. This period of time is almost same as the distance between the start point and the maximum chlorophyll level in prior phase therefore, this algae strain can assimilate and maximise photosynthesis within 4-5 days. After this time, the equilibrium between nutrient availability and chlorophyll production results in decline in pigments and we can say the starvation period has begun. The raises and falls in Chl-a content is a function of nutrient availability and time therefore we can control the trade-off between algal growth and chlorophyll degradation by adjusting the nutrient level and spent time. On 26th day, another proportion of urine with the same nutrient level (0.12 mg phosphorus) was added. The result was quick increase as it was expected. The important point about this maximum point is that by increasing the feeding time, the maximum level reaches higher values in compared to the last peak although the received nutrients were equal (Figure 23). For example, in U6 container which was fed 0.3ml urine 3 times, the Chl-a index raised to 19252, 52808 and 73080 respectively which shows more than 270% and 135% growth in each step. This means the maximum Chl-a and dry mass values after 3 steps feeding were about 4.9 mg/L and 160 mg/L respectively; whereas the initial concentration was less than 0.37 mg/L. It is expected to reach higher values by adding nutrient before decline starts, at maximum Chl-a level (every 4-5 days).

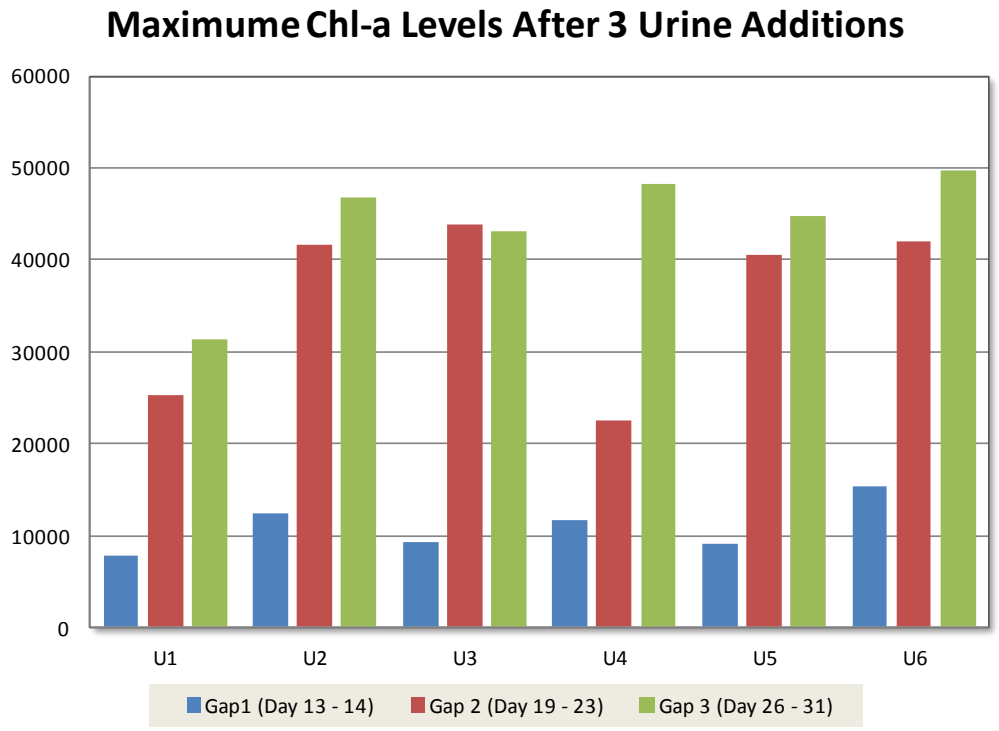

Figure 23- Increases in Chl-a levels after 3 consecutive urine addition (day 13, 19, 26)

This primary experiment has shown urine capability to handle autotrophic algae metabolism. Since the phosphorus level in both urine-fed and fertilizer-fed series was equal, presented growth diagrams have demonstrated that nutrients in urine are competitive and better proportioned for microalgae growth development than the artificial fertilizer used here. Moreover, urine feeding illustrated relatively high growth in microalgae production.

5.5. Experiment 6 - Algae Growth under High Urine Concentrations (Without Replication)

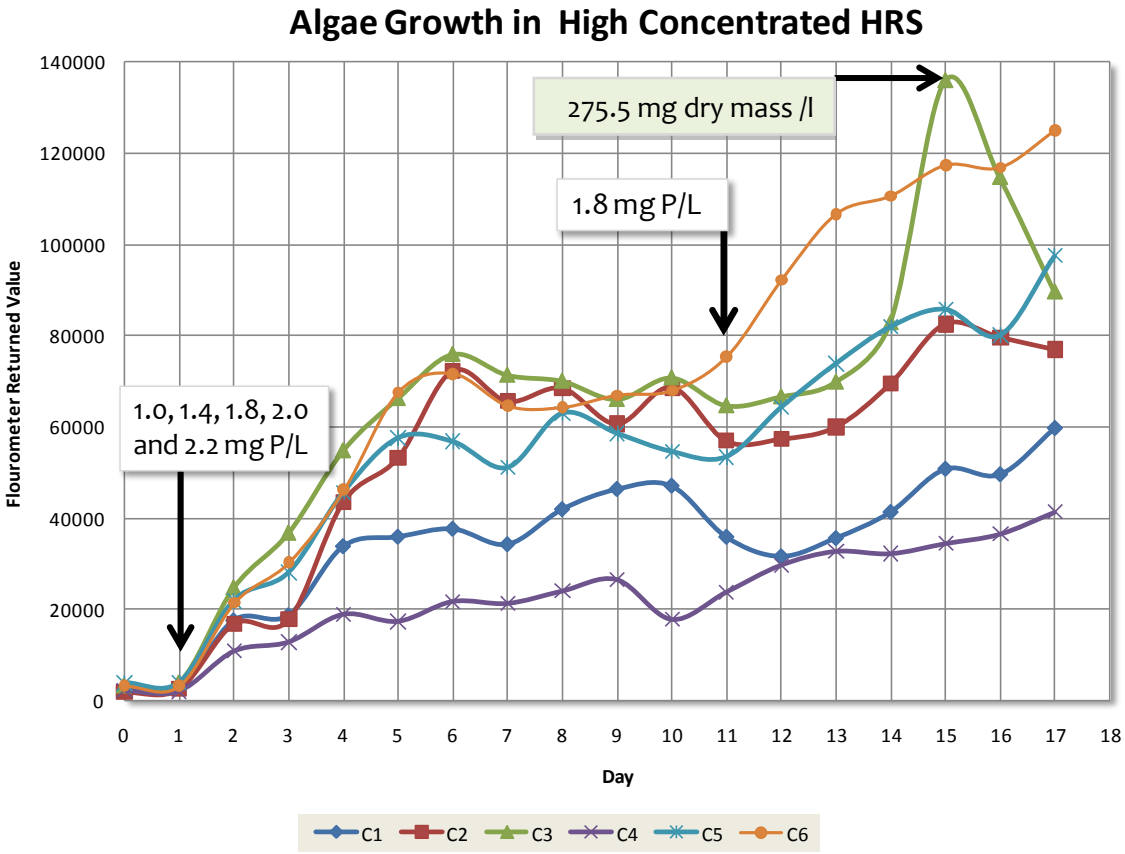

Figure 24- Growing under higher urine concentration solution. C1-C6 are representing growth in 6 containers with 1.0, 1.4, 1.8, 2.0 and 2.2 mg P/L . Respective amount of urine was added to each container at day 1. At day 11, all containers received urine equivalent to 1.8 mg P/L.

Urine was added one day after the algae were mixed in the balloons (Fig. 24). The initial Chl-a concentration was about 250µg/L and in first 5 days it became about 7035 µg/L in container 3 (over 2810% growth, Fig 25). These results are comparable with batch feeding result after 3 steps urine injection in lower concentration (Fig. 22). The general growth trends are in direct relation to added urine volume. Container 4 (C4) had the same problem then U4 had in last experiment and presented an unexpected growth as response to high nutrients level. For example, while the C3 with 1.8 mg P/L had the highest Chl-a value (7.03 mg/L) on day 7, C4 with 2.0 mg P/L was the lowest with 2.01 mg/L. The maximum biomass obtained on day 15, on C3 with 275.5 mg/L and growth rate of 17.9 mg L^{-1} d^{-1}. This experiment indicates that 1.4-2.2 mg PO_4 /L addition are optimum values.

If we consider C4 as an exception, the growth in the other containers was following the expectable model within the first 5 days. The important point about this experiment is that we did not experience serious decline after 5 days as occurred in previous experiments. The growth in most of the containers was fluctuating after gaining the maximum values but the general decline rare was not significant. The low decline can be caused by conversion of intercellular Acid-Insoluble polyphosphate (AISP) constituent to Acid Soluble polyphosphate (ASP) compounds in phosphorus deficiency [32][40]. Since the phosphorus accessibility was high initially, part of phosphorus supposed to be assimilated and stored in form of AISP luxury phosphorus uptake. After the phosphorus abundant reached critical level, this changed phosphorus restored to ASP form to be used in vital cellular activities and metabolism. This storage and release did not show up in previous experiences due to lower phosphorus availability and limiting AISP accumulation.

After 5 days, the second round of urine was added which gave a second increase in Chl-a level. The growth was maximized in C3 by 210% increase within 3 days (from 6.0 to 12.2 mg Chl-a/L). Although the production reached high levels, the overall growth was not as high as the first feeding. It can be due to lower phosphorus concentration in second feeding term and can relate to compensation in AISP storage which has been consumed between days 6 to 11. In this case the phosphorus abundance could not cause significant ASP synthesis and photosynthesis activities. Feeding urine for the second time in high concentration (over 2 mg/L) on 6th day can be considered as a solution for maximising the algal growth with the minimum time and urine consumption. In this experiment we reached the chlorophyll-a level up to 13 mg/L within 15 days and the dry mass on day 16 reached 0.314 g/L. It is expected to reach higher level of chlorophyll and biomass in shorter period of time by well-timed feeding under optimised concentration of urine. For example if the next feeding carried out with 5 days intervals with over 1.8 mg P/L urine content, we can expect harvest higher biomass and therefore higher biodiesel in supplementary phases.

5.6. Experiment 7 - Tap Water (TW) and Distilled Water (DW)-Based Cultures Comparison

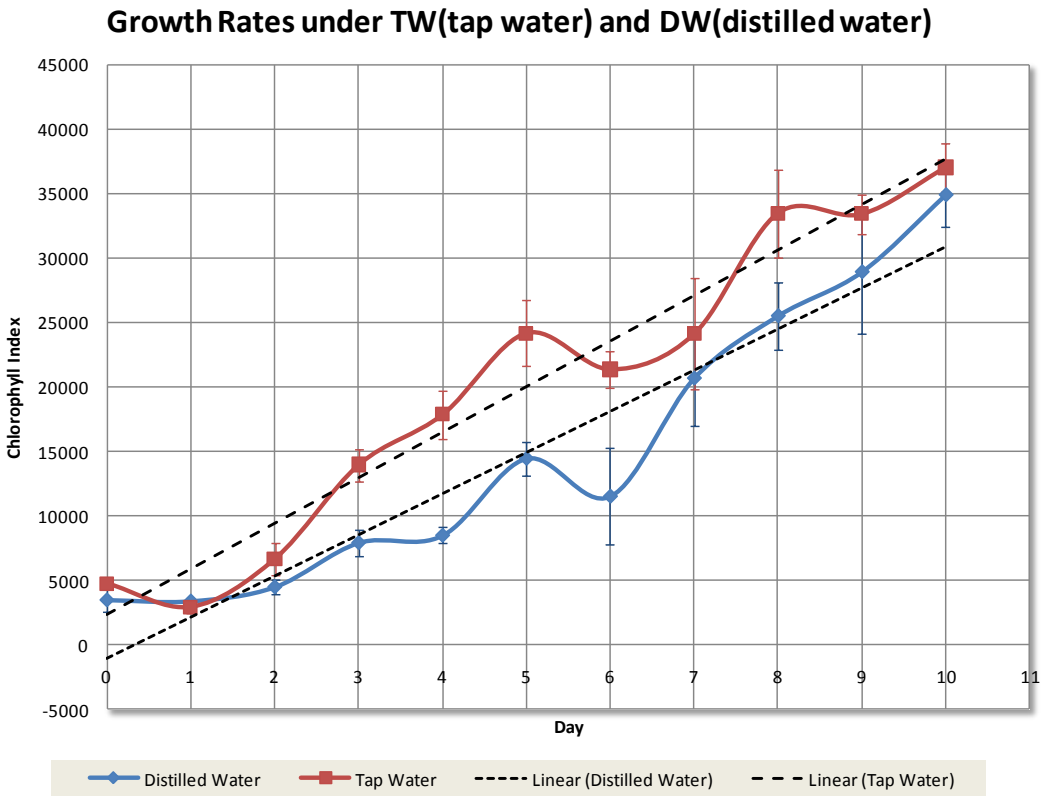

Figure 25- Distilled water(DW) and tap water(TW) growth (by series). The overall growth rate in TW series is higher than DW one.

Growth capacity in tap water-based culture is not unexpectedly higher than distilled water-based culture (Fig 26). The main difference between tap water and distilled (deionised) water, are minerals and trace metals ions which are to needed promote algae growth [1][42]. In Kristianstad drinking water, mainly these minerals are available according to *meteau* (2010) report [61].

Table 7 - Typical concentrations of some elements and ions in the Kristianstad city drinking water [61]

Parameter	pH	Conductivity	HCO_3	Ca	Mg	Fe	F
Unit		µS/cm	Mg/L				
Value	8	323	160	80	6.5	<0.01	0.35

Higher pH is better for algae cultivation (see Chapter 4 – pH section). pH of drinking water in Kristianstad is higher than 7.0 which we have in distilled water, therefore algae reach more proper condition to grow. One of the target applications of this study is promoting algae cultivation in city district scale to provide decentralized biodiesel production using the district residents' urine production. Therefore the water supply for localized photobioreactors can be drinking water or treated (UV disinfected) grey water which can accelerate algae production. The disadvantage of non-distilled water can be identified as phosphorus precipitation accelerator accompanying calcium, magnesium, iron or aluminium ions available in media, but as the graphs are demonstrating the growth rate supported by positive sides of drinking water is dominant compared to distilled water culture.

After about 7 days, the pH level in both series begun to reach similar levels due to primary production. More similar conditions caused the Chl-a values in both series to become closer after 7th day. Overall, tap water ingredients are recognized as positive factor for algal growth. Further studies on using recovered water (after algae harvesting) mixed with other fresh water sources needed to be carried out to make algae production more sustainable in large production scales.

5.7. Experiment 8 – Aeration Timing Effects on Algal Growth

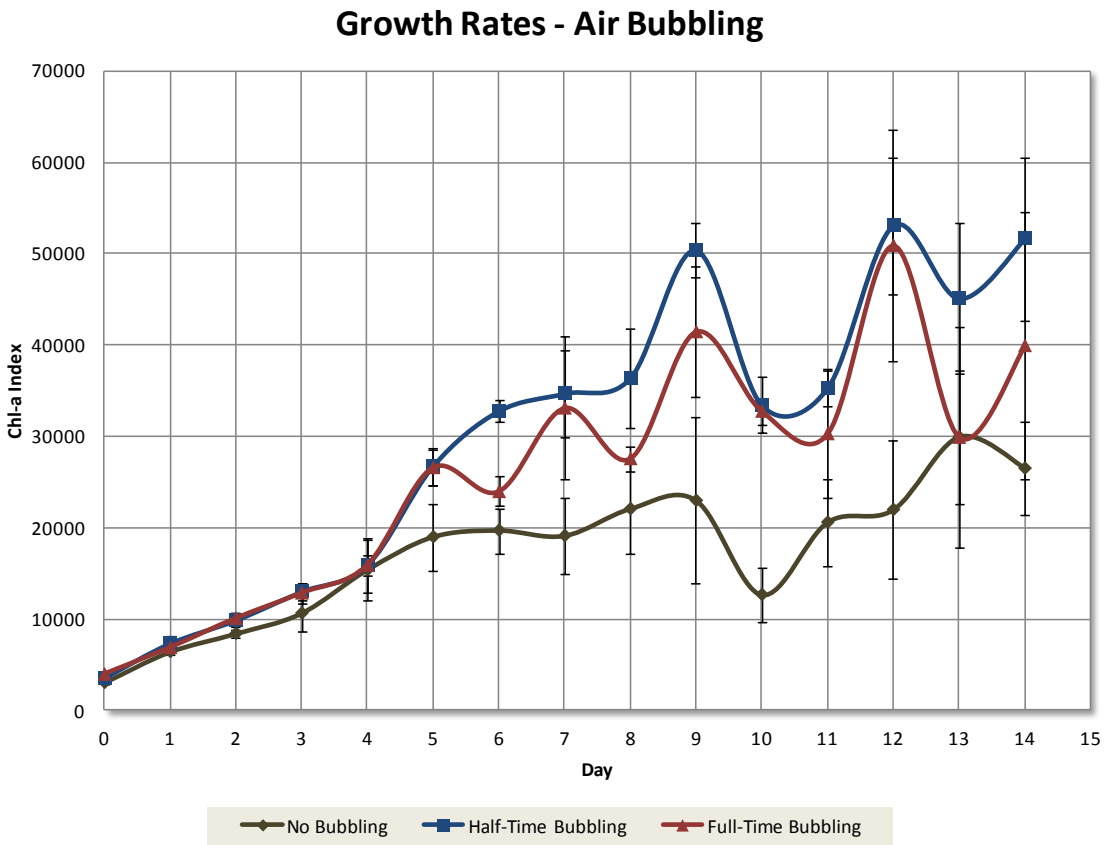

Figure 26- Growth diagram under different aeration timing. No bubbling (NB), Half-time bubbling (HB) and Full-time bubbling (FB) series

According to Figure 26, primary production in 50% (HB) and 100% (FB) aeration is higher than series without aeration. Aeration facilitates of algae-inoculums-atmosphere gas exchanges and accelerates CO_2 feeding and oxygen removal in the culture. Also, aeration prevents algae sedimentation and equal illumination to support photosynthesis. The HB series projects higher performance in comparison with FB samples. This can be interpreted as result of lowering the pH by aeration, increasing the CO_2 exchange. CO_2 in aquatic environment can release HCO_3^- and H^+ ions, leading to lowering the pH. On the other hand algal metabolism increases the pH and accelerates in higher pH levels (Figure 27). The pH in fulltime bubbling containers held the lowest pH levels compared to the other series, because the fulltime bubbling, constantly release H^+ and push the pH down which reduce the photosynthesis activities. The overall change of pH in the FB series is because of primary production influence on pH is dominant.

For the first 4 days of experiment we have similar growth in all samples. After day 3, sunlight was intensive enough to promote exponential growth after a period of cloudy weather (Fig. 26).

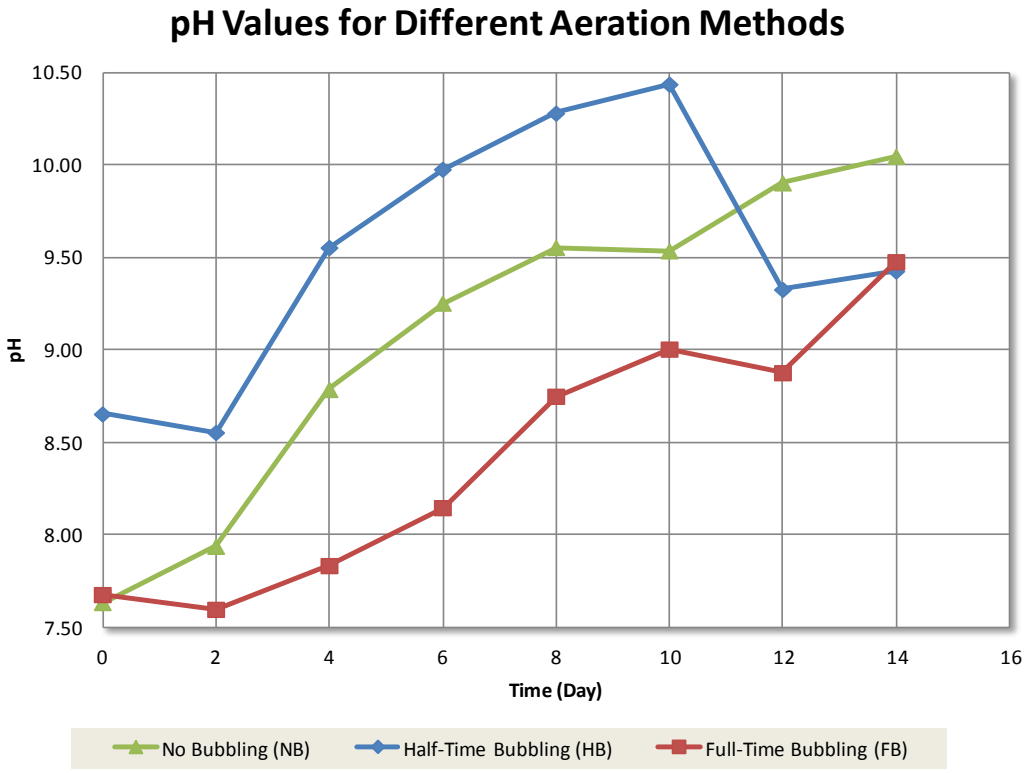

Figure 27- pH values in different aeration systems

In absence of nightly aeration, in halftime bubbling series, the pH reaches a higher levels which assist nutrients uptake and growth (Fig. 28). In the NB series, we have less but steady production because the algae cannot take advantage of aeration. The fluctuation in chlorophyll level in FB and HB series can be caused by change in light intensities during the experiment. When bubbling, light intensity differences can influence the whole volume whereas in NB series, a considerable proportion of culture stays in darkness due to self-shading specially at higher algae concentrations. Although the pH level of 8 is appropriate for most of the green algae species, different algae strains are adapted to different pH levels. The uncontrolled increase in pH level can cause growth prohibition. In this experiment, the pH in HB series increased to about level 10.5 due to primary production but the negative impact of this level of pH on *Scenedesmus quadricauda* decreased algae growth and consequently the the pH level dropped. This trade-off between algae growth and pH alterations can be considered as the reason to reach the same Chl-a level in both HB and FB series. This experiment has shown the positive effects of aeration emphasising the part time bubbling preferable supports for higher yield.

5.8. Experiment 9 – Comparison of Growth with Urine and Bold Media and Commercial Fertilizer

In this experiment, aeration, lighting, nutrient (phosphorus) level, temperature and algae strain was controlled. From the chlorophyll concentration curves, differences between urine series with Bold and fertilizer series in term of algae growth can be seen (Fig. 28, 30, 32). The overall growth in the urine serie (Urine 530) was distinguishably higher than Bold and Commercial Fertilizer series. We had exponential growth phase in urine serie (Fig. 28) and in the Bold's series (Fig. 30). In Fertilizer series, the exponential growth was absent and the algae entered stationary phase directly unpredictably (Fig. 32). The biomass level after 3 days up was about 2 times higher in Urine-530 (with dry mass of 39.98 mg/L) compared to Bold series (22.95 mg dry mass/L). This experiment has strongly proven urine capacities for *Scenedesmus quadricauda* cultivation compared to other nutrient rich sources. This can be interpreted by well proportioned nutrients levels in urine which is better for algae to uptake. High concentrations of macro nutrients in urine in addition to adequate micronutrients and proper pH level can be an explanation for this high growth rate. Also in urine, the major nitrogen components are in form of ammoniaum and urea which can be taken up by algae easily while the nitrogen in the fertilizer and Bold series is available in form of nitrate that is not assimilable as well [56][21].

In the Urine 530 serie, growth peak happened after 3 days (Fig. 30) compared to 5 days in earlier experiments. This can be as due to higher temperature (actual temperature +6 °C) and higher light intensity giving a higher growth rate compared to cultures grown in sunlight and room temperature. The initial dry weight was 16 mg L^{-1} and dry weight after 4 days was 91 mg L^{-1} averagely. For measuring the specific growth rate we can use these values in Equation 6 [16]

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \qquad (6)$$

Where x_2 and x_1 are dry mass concentrations at day t_2 and t_1 respectively.

In Urine 530 series, specific growth rate was 0.58 mg L^{-1} day $^{-1}$. According to experiment plan, fresh urine with 0.53 mg/L phosphorus value added to Urine 530 at day 5 and on day 7. Increase in Chl-a happened in all samples. Since urine was added 2 days after the maximum point, algae experienced nutrients depletion generally and this caused algae idle mode to some extent. Therefore the responses to new nutrients, was only high Replica #1 (Fig. 28). This replica responded positive to urine addition and increased 100% more than other replicas. It is supposed all replicas would behave like replica #1 if re-nutrition was done on day 3. The average dry mass at day 9 was 94 mg L^{-1} while it was 135 mg L^{-1} for replica 1. This alteration in algae behaviour caused a significant decline in growth rate after day 4. For this period of time, the average growth rate calculated was 6.5 mg $L^{-1}d^{-1}$ from day 4 to day 9 while it was 79 mg $L^{-1}d^{-1}$ for replica 1. It is very important to utilize urine batch feeding on the proper time to reach the highest biomass yield.

In Bold series, the initial peak was between day 3 and 4 (Fig. 32). The grow rate was not comparable with urine samples. Initial dry mass was 13 mg L^{-1} and after 4 days 33 mg L^{-1}. For Bold's series, the specific growth rate was 0.31 mg day $^{-1}$. The Chl-a was decreased after day 4. At day 5, 200ml of nutrients solution was replaced with 200 ml of culture in all vessels but no changes was observed in the Chl-a values and the series was shut down on day 7.

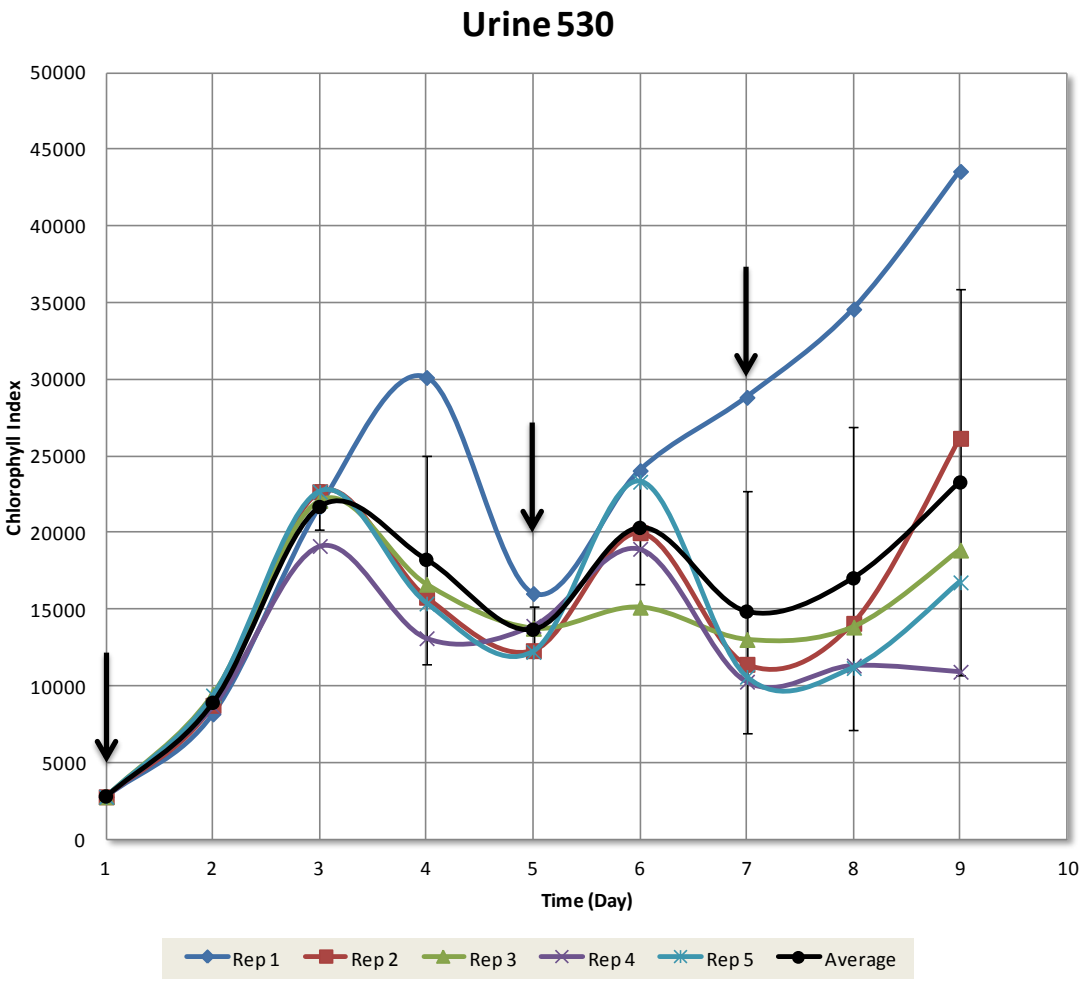

Figure 28- Growth curves by urine feeding (0.53 mg Phosphate-P/L) in 5 replicas (Rep1 – Rep5) and the average growth and standard deviations. Black arrows are indicating the feeding rounds.

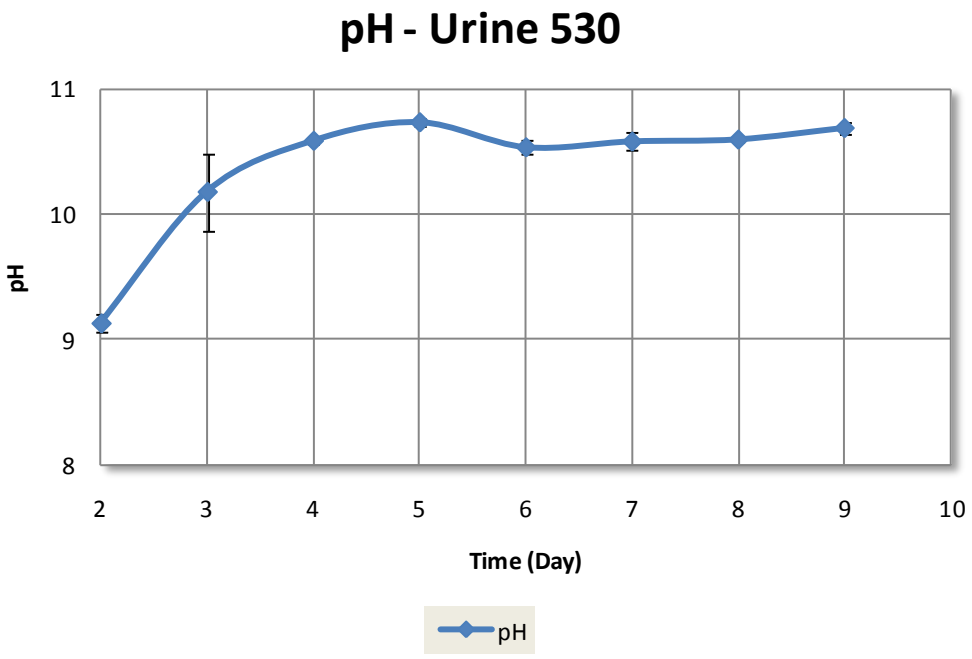

Figure 29- pH variations in Urine530 series

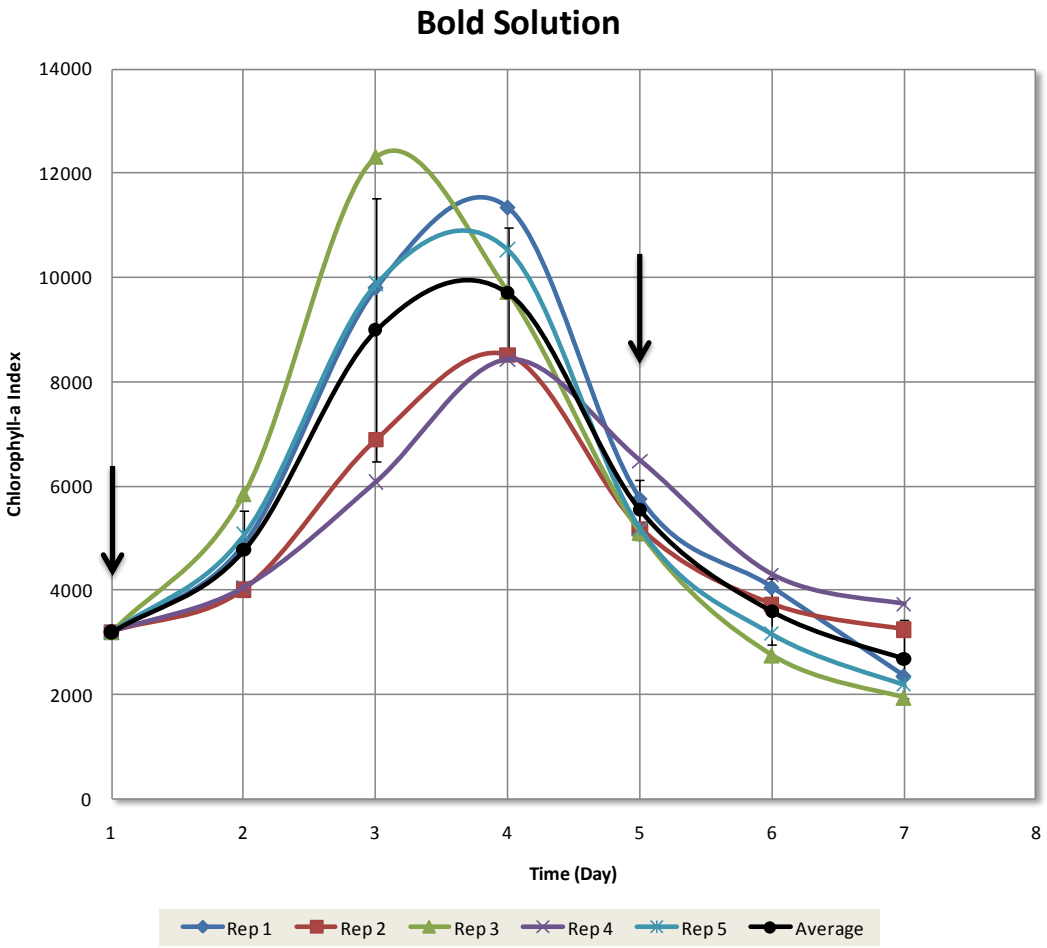

Figure 30- Growth curves under Bold's solution feeding (0.53 mg Phosphate-P/L) in 5 replicas (Rep1 – Rep5) and the average growth. Black arrows are indicating the feeding rounds.

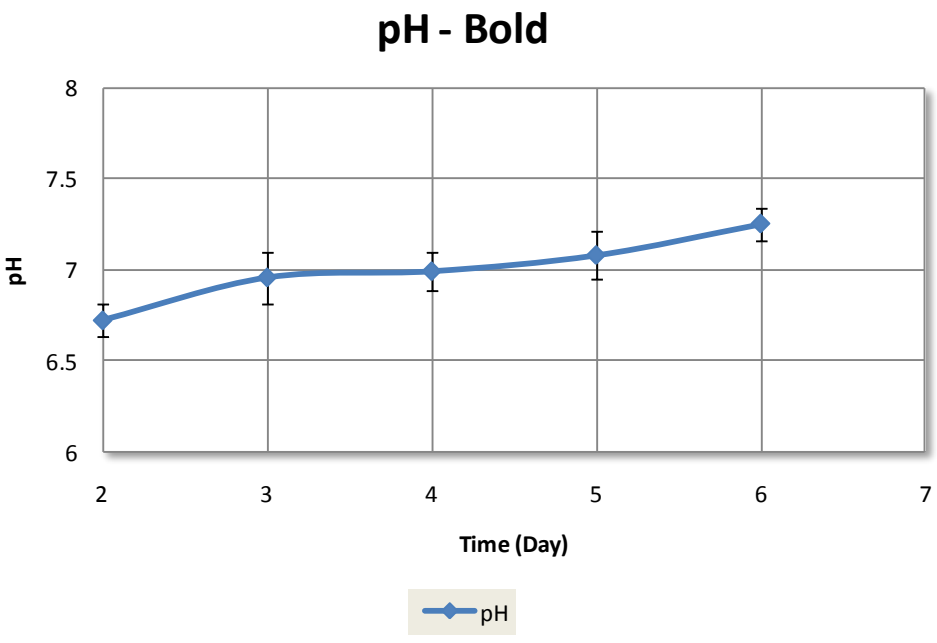

Figure 31- pH variations in Bold series

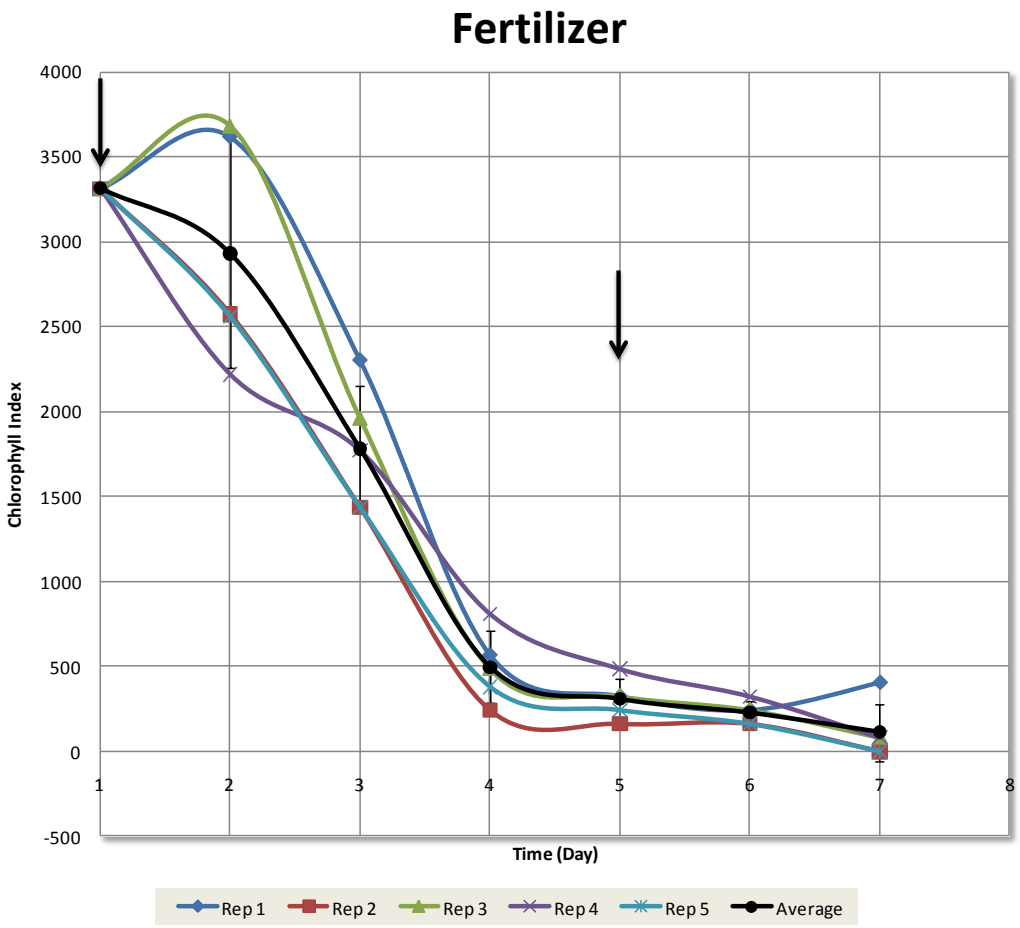

Figure 32- Growth curves by fertilizer feeding (0.53 mg Phosphate-P/L) in 5 replicas (Rep1 – Rep5) and the average growth. Black arrows are indicating the feeding rounds.

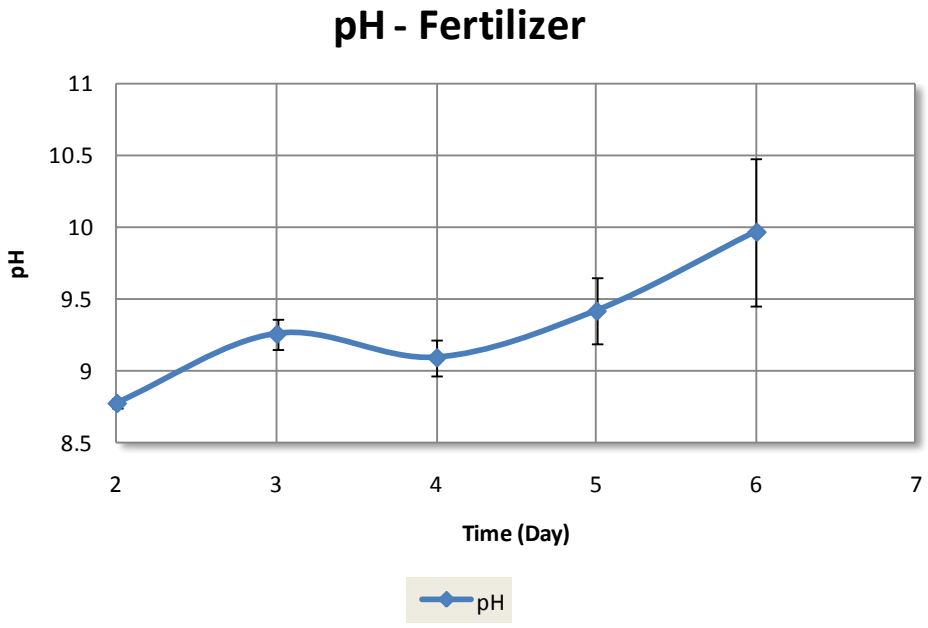

Figure 33- pH variations in Fertilizer series

In Fertilizer series (Fig. 32), the results were different compared to the Bold series. In this series, no overall increase was recorded in chlorophyll-a production. This series had a very sharp drop in Chl-a level and entered to the idle phase. Even after fertilizer addition for the second time day 5, no recovery was observed. The initial dry mass concentration was 13 mg L^{-1} and the measured dry mass on day 4 was 0.8 mg L^{-1} (μ= -0.93 mg $L^{-1}day^{-1}$). Since this fertilizer is nutrients rich, no explanation due to nutrients deficiencies can be provided. The possible reason for this growth failure can be due to high level of ammonia accumulation which can cause growth inhibition [1][2].

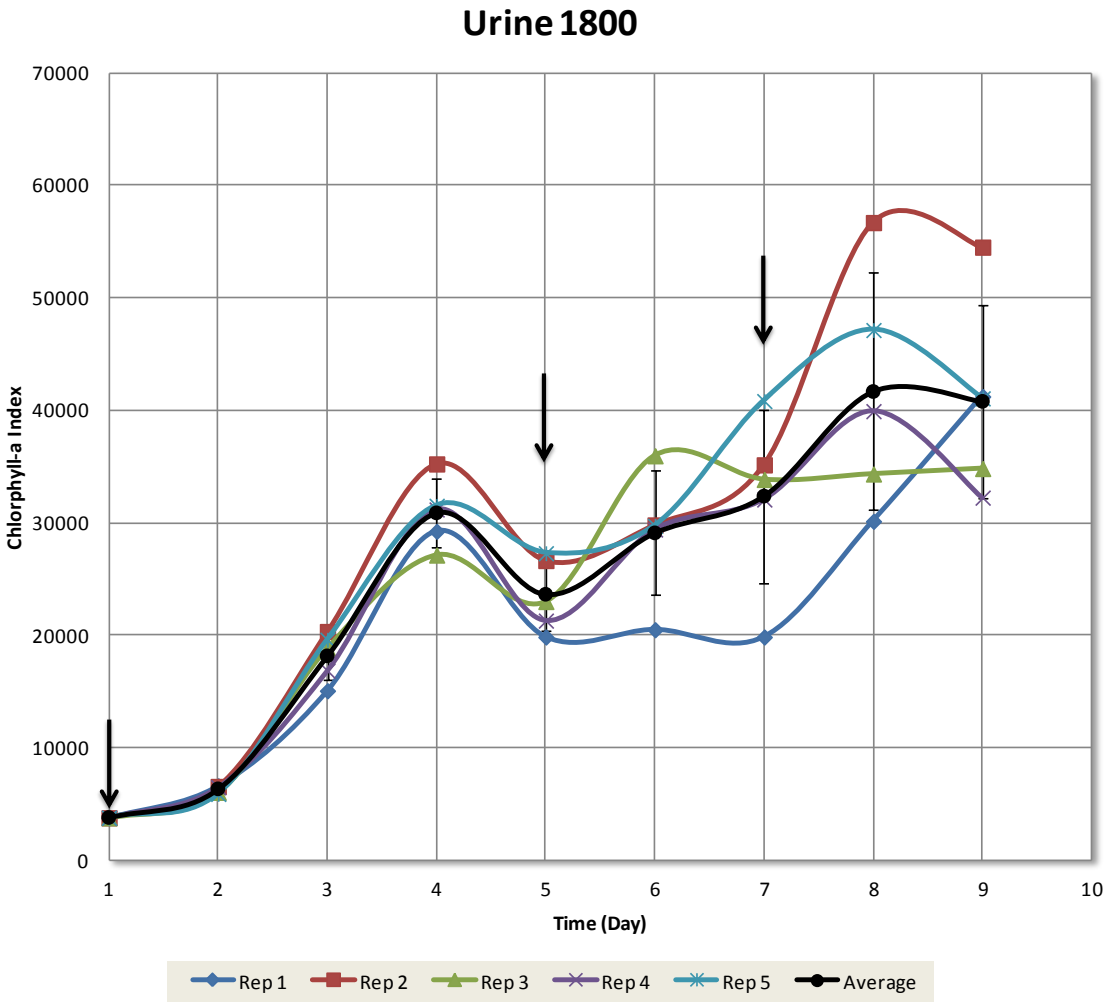

Figure 34- Growth curves under urine feeding (1.80 mg Phosphate-P/L) in 5 replicas (Rep1 – Rep5) and the average growth. Black arrows are indicating the feeding rounds.

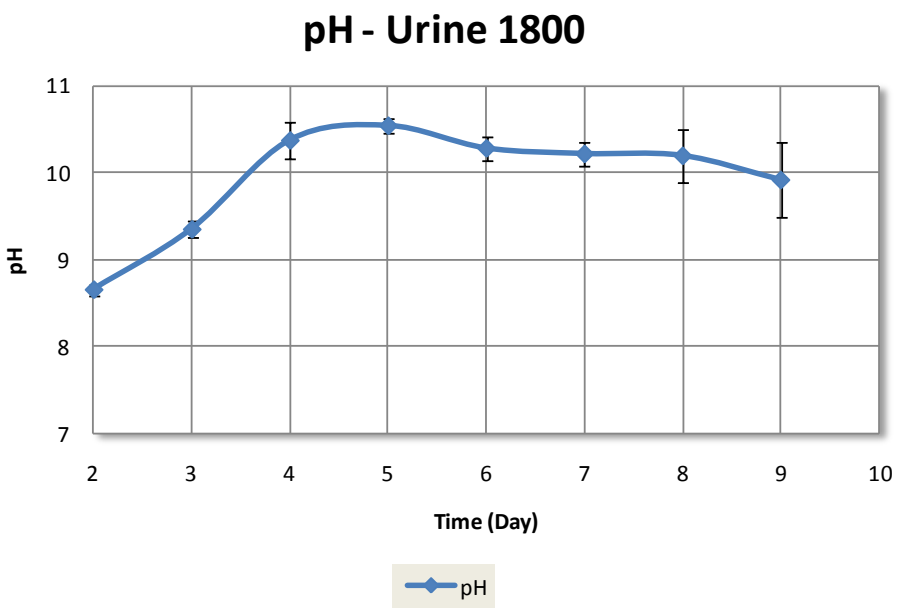

Figure 35- pH variances in Urine 1800 series

It was shown that a higher urine concentration, gives a higher the yield achievable (Fig. 36). In Urine 1800 series, the higher nutrients concentration gave a higher exponential growth with higher maximum Chl-a index compared to Urine 530. The lag phase was longer and the growth was supported till day 4 (1 day more than urine 530 series). The nutrients recharging responses was higher and fewer replicas entered to idle state compared to Urine 530 series. In this series, replica 1 and 3 can be considered as idle. The grow rate in this serie was higher than all other series. The initial dry mass concentration for this serie was 17 mg L^{-1} and the average dry mass at day 4 was 115 (±9.56) mg L^{-1} (μ=0.64 mg L^{-1} d^{-1}). The replica #2 was the most productive sample with 142 mg L^{-1} yield on day 9.

In Figure 36, the average Chl-a levels for the four nutrient sources are presented. The growth is supported by batch feeding but the growth rate is lower than the initial rate. Therefore it can be assumed by increasing the initial concentration, we can reach higher peaks and then by supporting the growth with less nutrients exactly before the decline starts, we can optimize the production.

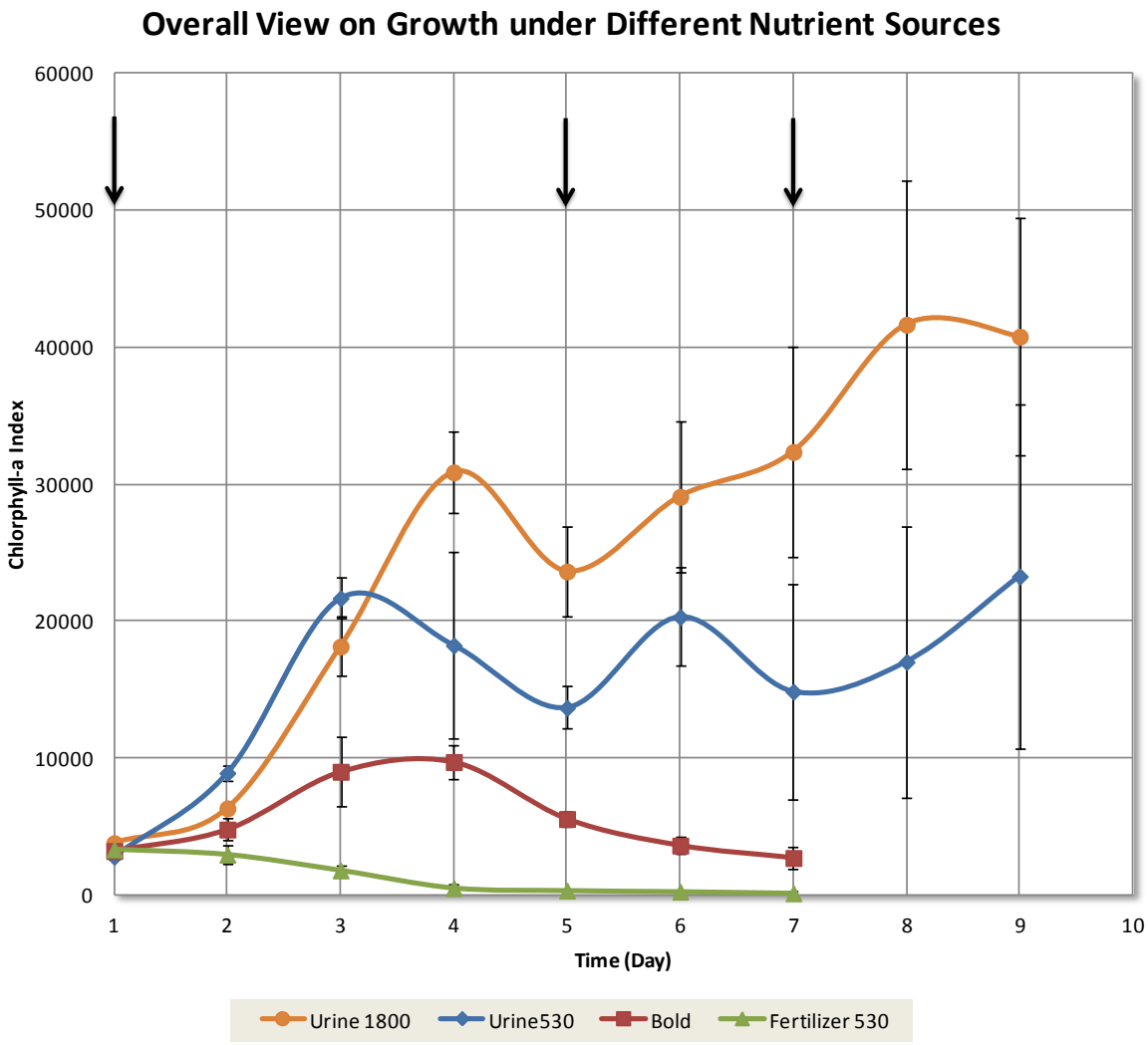

Figure 36- Overall increase in growth measured as Chl-a under different nutirent sources

By increasing the urine, we can reach the promising increase in algal biomass but the optimum urine concentration should be investigated. Also the production upon batch feeding in form of once high urine concentration and then continues but daily portioned of urine from the peak (day 4) is suggested to be assessed.

By measuring the Total-Nitrogen (Tot-N) and Total Phosphorus (Tot-P) of filtered culture on day 1 and day 5, the nutrients availability and removal was determined. The initial Tot-N concentrations were 11.45 and 41.3 mg N/L and the initial Tot-P concentrations were 603 and 1783 µg P/L in Urine 530 and Urine1800 series. After 5 days, the average Total-N in Urine 530 and Urine 1800 was 4.42 and 28.08 mg N/L and the Tot-P concentration 21 and 82 µg P/L respectively (Figures 42-45).

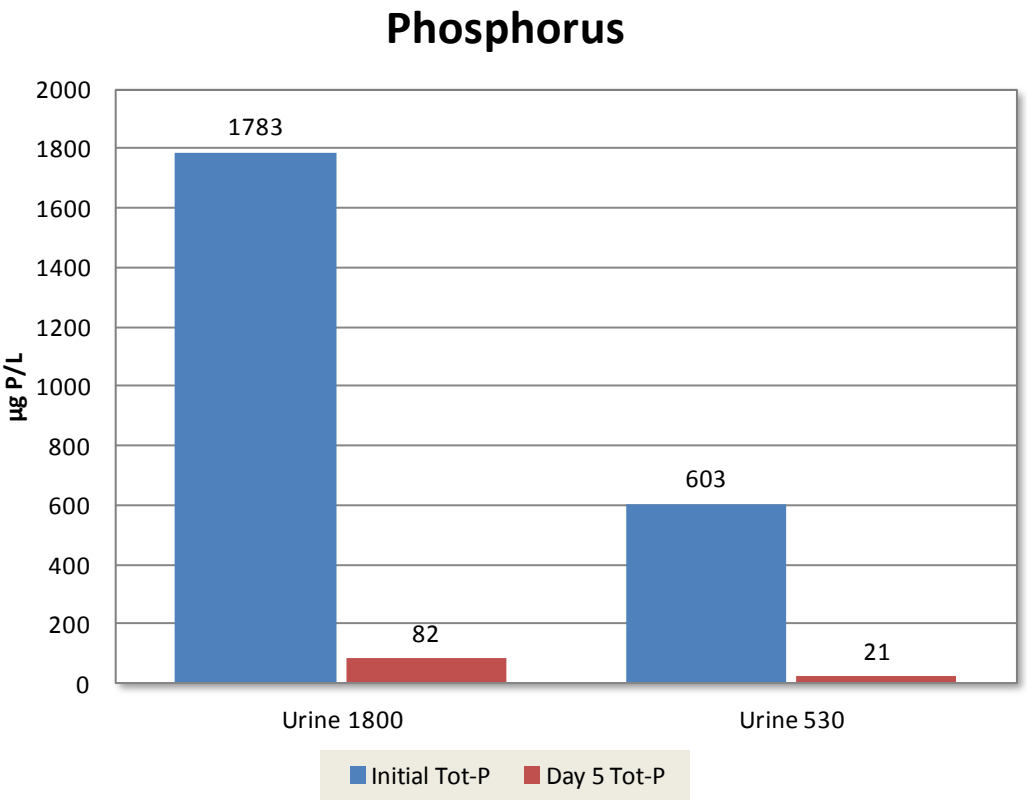

Figure 37 - Phosphorus level in culture, at start day and after 5 days (values in µg P/L)

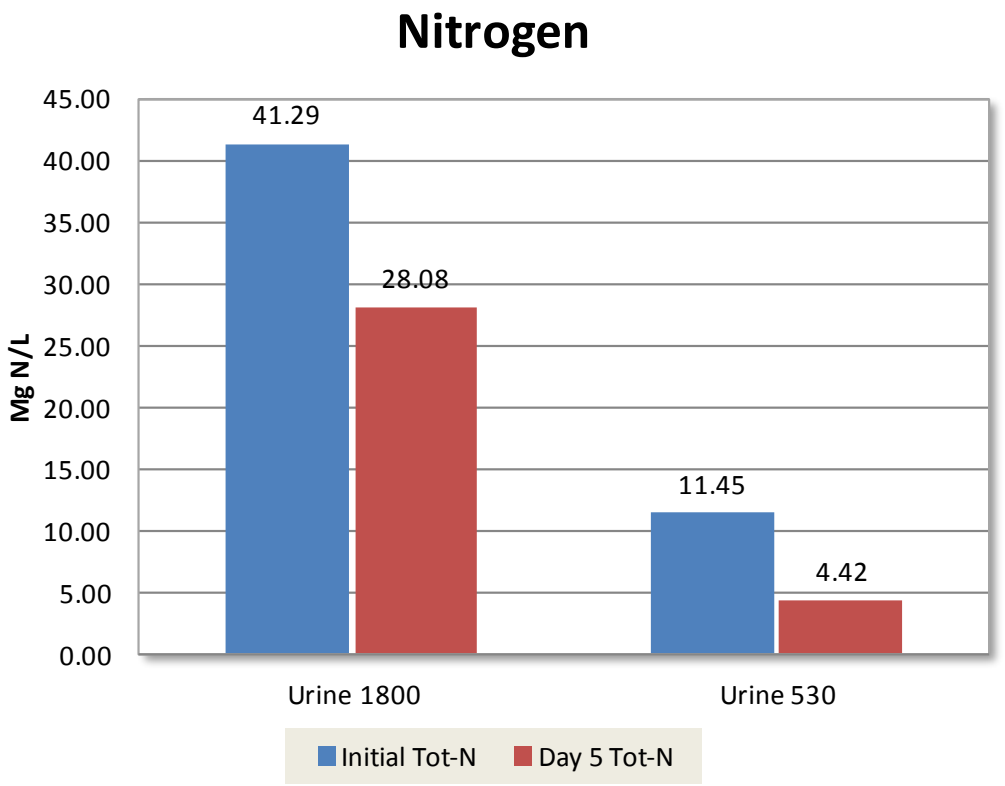

Figure 38- Total Nitrogen levels in culture, at start day and after 5 days (Values in mg N/L)

According the phosphorus and nitrogen levels in culture medium, it is obvious phosphorus is the limiting factor in algae production (Fig 43, 45). Over 95% of the phosphorus was removed compared to 60% for nitrogen. This fact leads to high concentrations of nitrogen at end of each growing period. By extending urine feeding, the leftover nitrogen ratio gets higher which can cause osmotic pressure on cell membrane, and cause problem in leftover phosphorus uptake. Also the growth inhibition due to ammonia accumulation next to high levels of pH can be another factor preventing biomass increase [1][2]. According to these results, N:P uptake ratio of 7.77 to 12.09 is applicable by *Scenedesmus quadricauda* within the examined range of phosphorus availability (603-1783µg P/L). Therefore higher ratios (caused by higher nitrogen abundant in urine) will be remained unassimilated. Urine in this experiment consists of nitrogen/phosphorus ratio of 32.9 (6255N/190P mg/L) which is about 3 times higher than the value we had in urine 530 series. To support steady algae growth it is needed to maintain the N:P ratio in specified range. By time, this ratio was changed to 342 which is very higher than the optimum range of 20-30 for algal reproduction [62]. This nitrogen level is not desirable since we need to give the algae nitrogen stresses via nitrogen depletion for increasing the lipids production in form of Triacylglyceride(TAG) molecules [17][3][21][42]. This experiment also shows high nitrogen level in urine is not well proportioned for promoting algal. To solve this problem, we need to either

1- Remove nitrogen from urine prior to algal feeding
2- Remove the nitrogen from the culture after reaching the certain targeted biomass yield
3- Enrich the urine with extra phosphorus resources to equalize the phosphorus and nitrogen efficiency in algal growth and reaching the nitrogen and phosphorus depletion spontaneously when the biomass yield is maximized.

As a suggestion, wastewater after secondary treatment (prior phosphorus removal in tertiary treatment) can be used to dilute urine and also increase the phosphate-P values of solution and decrease the N:P ratio to promote efficient biomass yield and also lipids accumulation.

For alterations in urine compounds or increasing nitrogen removal, more research is needed.

5.9. Experiment 10 – Assessment of High Urine-Nutrients on Algae Growth

In this experiment high urine levels were tested as growth media for the algae, the overall result of this experiment is that the high nutrients availability (in range of 2.5 mg P/L and above) is not favourable for algae production in batch feeding method. The decline in U-3.0 and U-3.5 was more apparent. In U-2.5, lower growth was observed than U-2.0 (Fig 39,40,41,42).

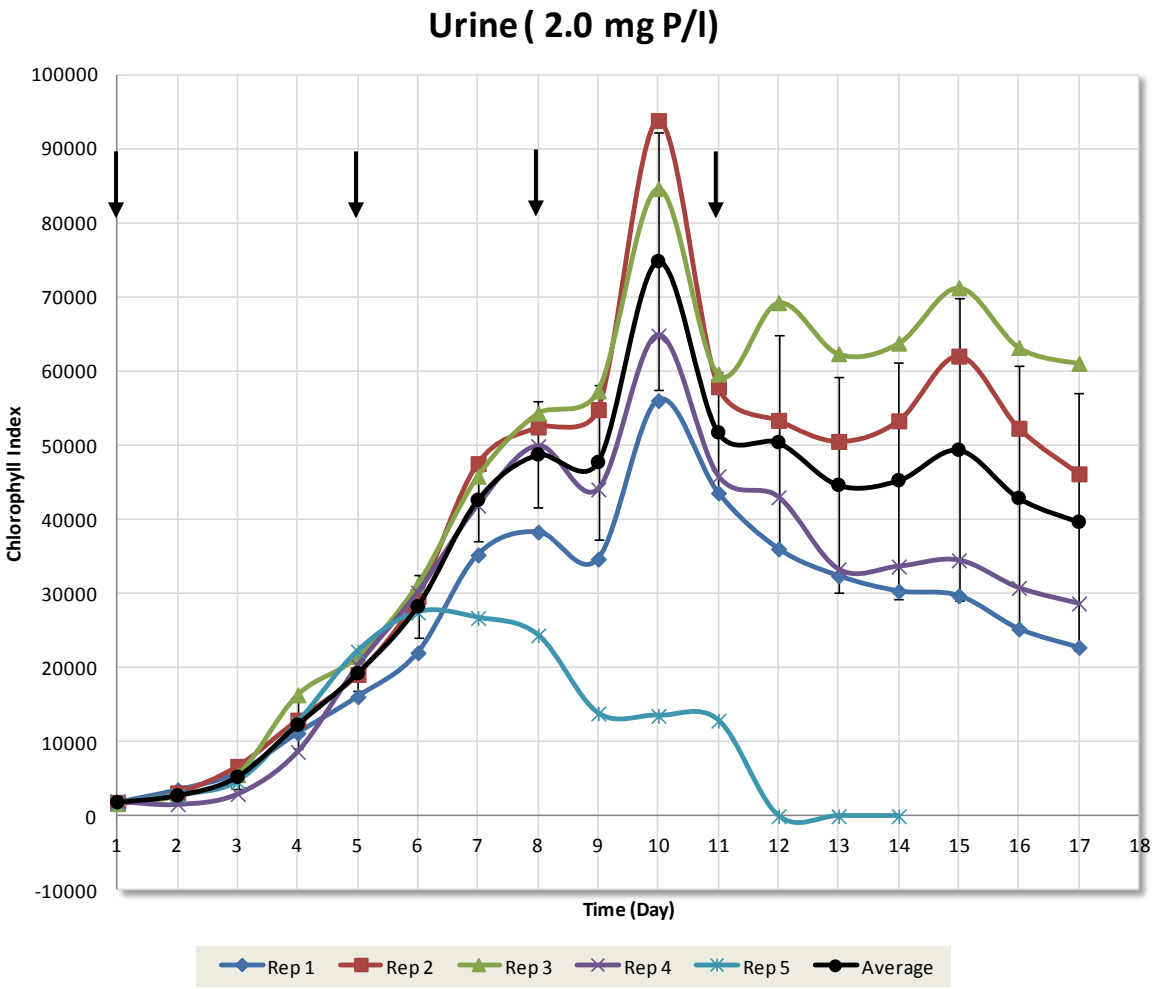

Figure 39- Growth in 2.0 mg P/L urine series. Rep-1 to Rep-5 are replicas with the same environmental and nutrients conditions. The black arrows are indicating the urine feedings

In Figure 39 we can see that the variations in series increases by time. In Replica 5, unexpected decline appeared which was following the idle growing mode therefore this replica has excluded from average calculations. The first feeding time (at day 1) has supported the exponential grow and seems next feeding time (at day 5) has supported this growth. Since the biomass increase should be associated with nutrient abundance to maintain the steady growth rate, the next feeding took place on day 8 but to reduce the nutrients shock, the urine injection was divided into two stages on days 8 and 11. But after feeding on day 8, the algae growth recorded a decline on day 9 and a sharp increase in day 10. This growth can be caused by good phosphorus repletion and also received minerals available in fresh tap water (as explained in 4.2.7). The maximum biomass achieved was 153.2 mg/L averagely on day 10 with growth rate of 16.2 mg $L^{-1}d^{-1}$.

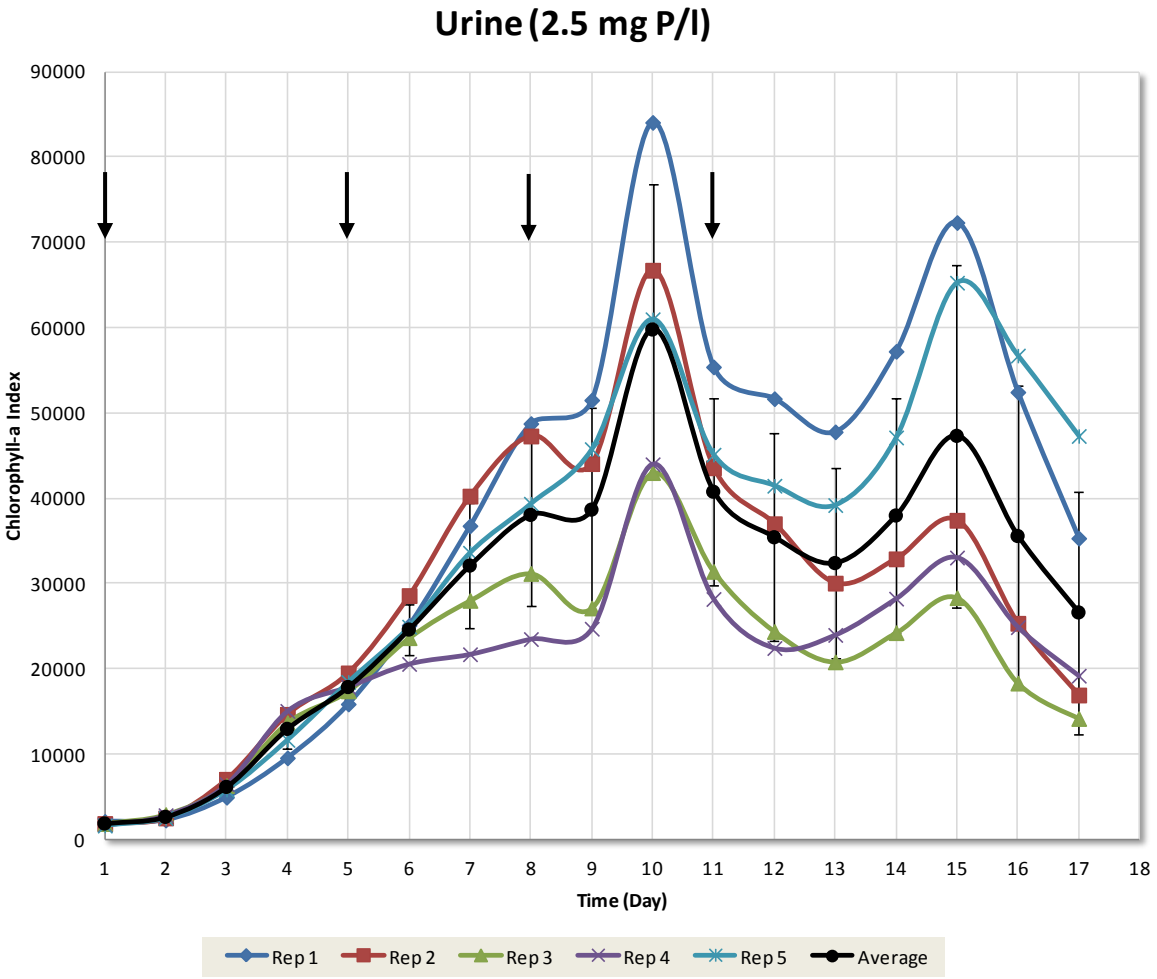

Figure 40- Growth in 2.5 mg P/L urine series. Rep-1 to Rep-5 are replicas with the same environmental and nutrients conditions. The black arrows are indicating the urine feedings

The growth in 2.5 mg P/L (Fig 44) is similar to 2.0 mg P/L but peak values are lower. The diluted urine feeding seems to be more effective in U-2.0 urine series. It can be caused by bigger differences in extra nutrients availability and also preventing ammonia inhibition. On day 10 we reach the highest yield as a response to phosphorus abundant in the medium but from day 11, the Chl-a level declined. This decline may be caused by low phosphorus high nitrogen availability as explained in Experiment 9. The next peak happened on day 15, four days after second proportion of third feeding takes place. In this conditions, the N/P ratio and also the biomass concentration are very high therefore by adding new urine, the phosphorus cannot support increase in biomass for a long time and the temporary decreased N/P, rolls back to high ratios with fast P assimilation by algae.

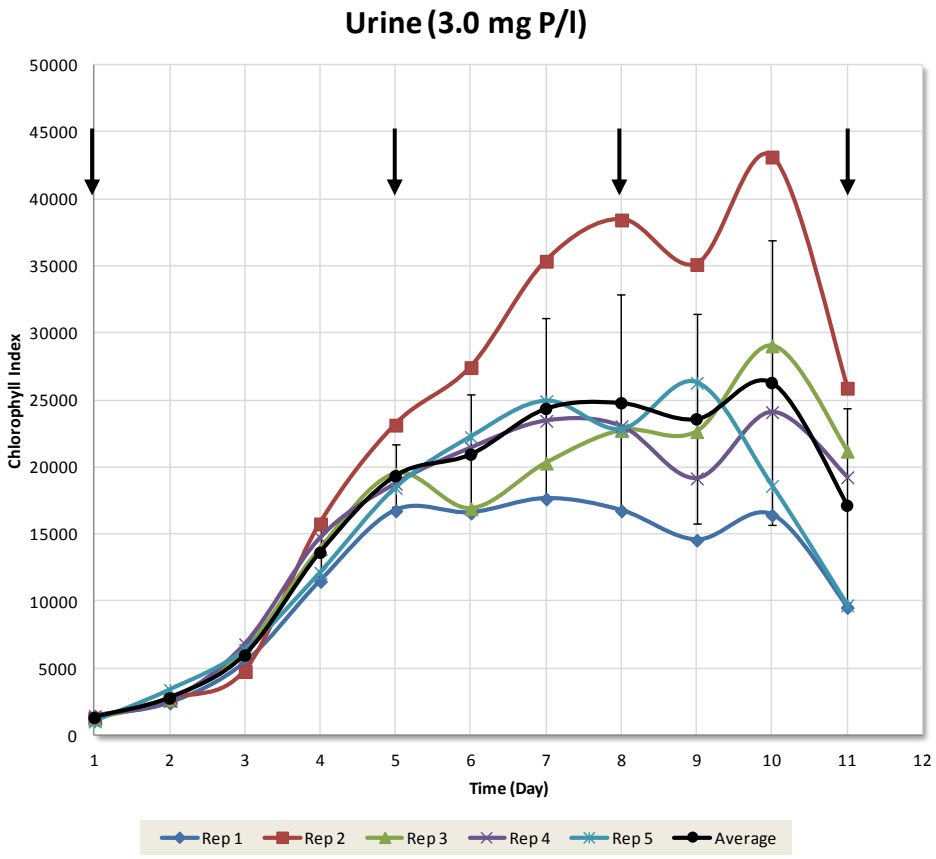

Figure 41- Growth in 3.0 mg P/L urine series. The black arrows are indicating the urine feedings

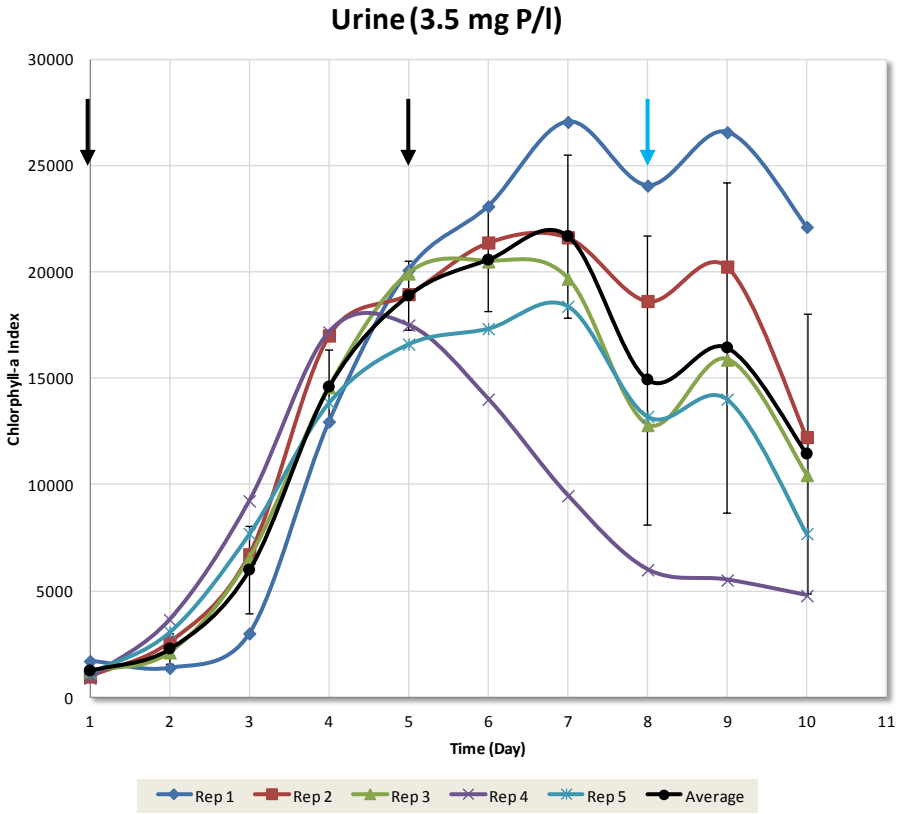

Figure 42- Growth in 3.5 mg P/L urine series. The black arrows are indicating the urine feedings. Blue arrow shows when 200 ml of culture was replaced with 200 ml of tap water.

In U-3.0 and U-3.5 we have a similar trend in growth (Fig 45, 46). The exponential growth rate was almost the same for the all series till day 5, but after the second feeding on day 5, the overloading in nutrients availability caused growth inhibition in 3.0 and 3.5 mg P/L series (Fig 47). Obviously the higher productivity in U-3.0 is connected to lower nutrients content which is following the same trend in U-2.0 and U-2.5. In U-3.0 series, the exponential growth stopped after second feeding at day 5 which means the nutrients shock might prevent algae growth in this series. It is supposed the nutrients consumption in all series is the same before refeeding at day 5, therefore the nutrients concentration after this day is much more in high urine concentration series (U-3.0 and U-3.5) than in the others. In this case, the adding more nutrients at day 5, can be growth supportive for less concentrated series (since the phosphorus level is low after uptake during 5 days) and be preventive for 3.0 and 3.5 mg P/L series since the N/P ratio becomes high which is not favourable for algae culture [62].

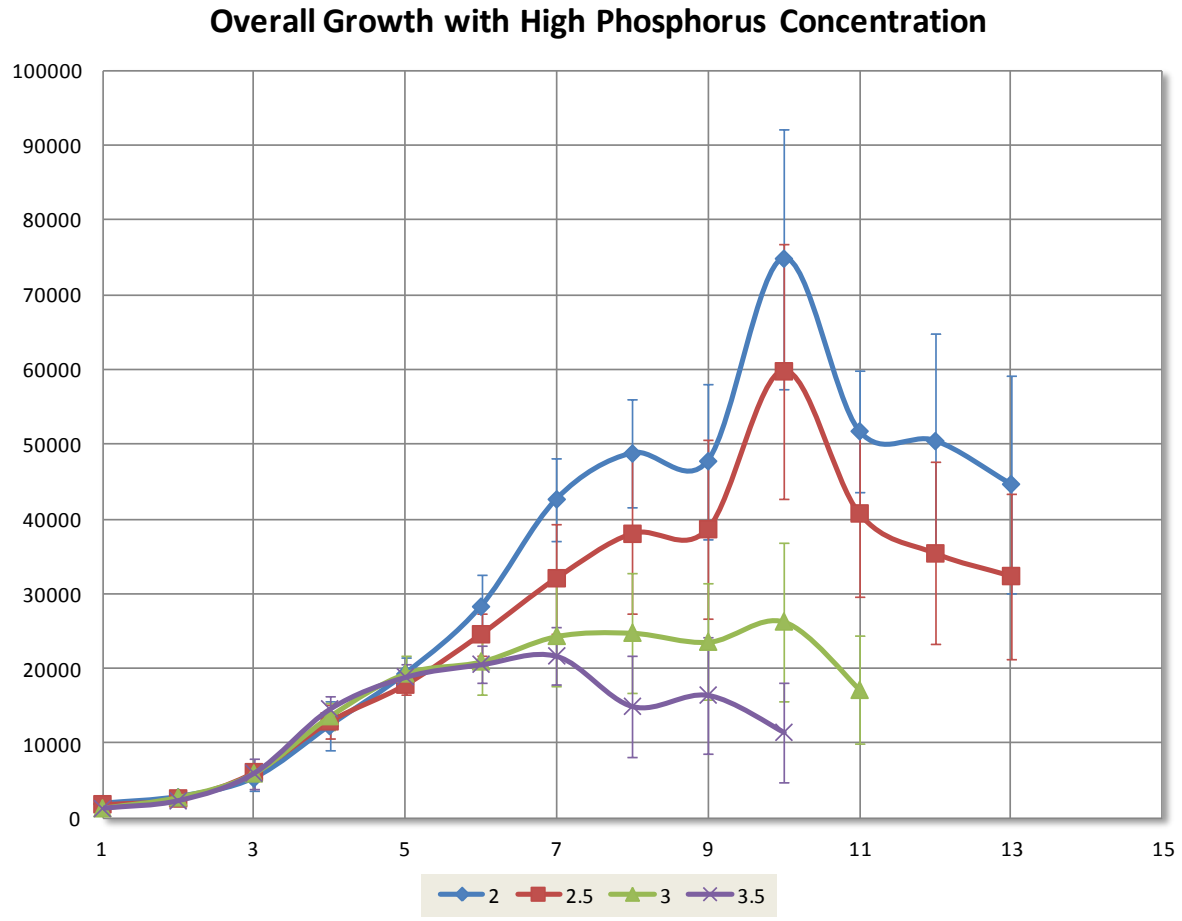

Figure 43- Overall growth patterns in 2.0, 2.5, 3.0 and 3.5 mg P/L urine series

In Figure 43, it can been seen that high concentration of phosphorus in microalgae culture will not lead to higher algae culture because of higher P and N availability. According to our results, it can be assumed that the maximum phosphorus concentration in urine for *Scenedesmus quadricauda* is about 2.0 mg P/L. Moreover the composition of urine, feeding methodology and timing are important in supporting the algae growth. For example it is assumed that in U-3.0 series, if the feeding interval was more than 4 days, (between days 1 and 5), the Chl-a level would be higher as well. Furthermore the batch feeding is assumed to be more efficient due to providing as much nutrients as needed in every growing stage. Since the available equipments was not enough for running semi-continues feeding, it is impossible to compare current batch feeding method (high nutrients injection in every feeding time) with continuous feeding with low hydraulic retention time (HRT) and coupled with dilution by tap

water to support more minerals availability. Every day feeding with incremental rate (fed-batch) could be better feeding methodology than the performed feeding schedule [63][64].The algae should not have more nutrients than they can use, and nitrogen should be removed after certain periods of time to maintain the N/P ratios (20-30). This can take place through hydrolysis or nitrifying (to prevent ammonia accumulation) the culture to degrade urea and ammonia.

5.10. Conclusive Discussion

For Scenedesmus quadricauda, the lipid storage capacity is relatively low (35% DW) whereas some other species (e.g *Chlorella pyrenoidosa*) have shown high lipid accumulation up to 64% DW. Also the doubling time is Scenedesmus sp. is higher than lipids rich species like Chlorella. Doubling time has significant role in feasibility study of strains as lower doubling time, leads to higher biomass yield in certain period of time. It is important ro mention it has been strongly shown that high lipids production takes place under N-deficient condition for chlorophytes (green algae) [65]

Microalgae *Scenedesmus quadricauda* is not a preferable algae stain for lipids accumulation because the lipid content and the growth rate is relatively lower than some other highly productive strains[66]. For example it has been reported that *Chlorella* sp. with maximum biomass growth rate of 0.37–0.53 g L^{-1} d^{-1}, contains 32.0–34.0% dry content weight (DCW) of lipids with lipid productivity of 121.3–178.8 mg $L^{-1}d^{-1}$ where as these values for *Scenedesmus quadricauda* are 0.19 g L^{-1} d^{-1}, 18.4 % DCW and 35.1 mg $L^{-1}d^{-1}$ respectively [51]. In Experiment 9, the maximum dry mass of 153.2 mg/L was obtained after 9 days with biomass growth rate of 16.2 mg L^{-1} d^{-1}. In experiment 6, the biomass yield of 275.5 mg L^{-1} was achieved within 15 days by growth rate of 17.9 mg L^{-1} d^{-1}. *Scenedesmus sp.* is an appropriate algae for carbon capture [67]; therefore our relatively low values in biomass production can be caused by lack of CO_2 in culture although the aeration was done during the experiments. Moreover performance of lipids extraction and processing methodology toward FAME production is very different based on the technology usage [51]Therefore we can expect very different biodiesel yield under certain nutrients and environmental conditions upon using different algae species and harvesting, extraction and processing technologies. Rawat *et al* has stated that 63.9% of the obtained algae oil is convertible to biodiesel. [46]

If we assume we need 5kg of biodiesel to run a car for 100 km, and the lipid to biodiesel conversion efficiency be assumed 64% (There are solutions to reach up to 98% efficiency[51]), therefore we need 7.81 kg of algae oil (in form of TAG mainly). And if we have 17 mg L^{-1} d^{-1} *Scenedesmus quadricauda* biomass production (as an average of results of Experiment 6 and 9), and the lipids level of 18.4 % dry DCW, we need 42.45 kg (dw) of algae which means we need 1783600 L of culture for 14 days. If the phosphorus concentration in urine be assumed 420 mg P/L (the concentration we had in our experiments), to produce 2.0 mg P/L culture, we need 4.76 ml of urine per 1L of culture medium. Then we need 8490 L of urine to run the car for 100 km.

If we cultivate Chlorella *sp.* with biomass growth rate of 0.45 g L^{-1} d^{-1}, contains 33.0% DCW of lipids with lipid productivity of 150 mg $L^{-1}d^{-1}$, after 14 days, we will have 2100 mg lipids L^{-1}.Therefore to produce 42.4 kg of lipids, we need 20200 L of culture which means (with 2.0 mg P/L content) 96 L of urine is needed. This great differences show the effects of selecting algae strain on biodiesel production.

However according to our results, the urine should be manipulated to optimize the suitable N:P ratio for the algae growth. Using refined urine (reduced in nitrogen level) by offgassing the volatile ammonia or enriching urine with external phosphorus sources (e.g. cheese whey) can significantly improve the culture quality in term of enhancing N:P ratio and avoiding ammonia inhibition as main negative points of using urine for microalgae culture.

CHAPTER 6

CONCLUSION

Biodiesel has been recognised as one of the promising renewable energies. Third generation of biodiesel is the most sustainable biodiesel which relies on algal growth and oil storage. The main resources for promoting algal growth are nutrients, light and carbon dioxide. One of the most important non-renewable nutrients is phosphorus. Human urine is enriched with nutrients (including phosphorus) which can support algal growth. As seen in this study, urine performance in algae culture has been compared with other chemical fertilizer and culture solutions (a commercial fertilizer and Bold solution). In all of the experiments conducted, urine performed better than other nutrient sources, which proves its capability to replace with conventional fertilizers. Batch feeding, with high concentration of phosphorus in urine is recommended to reach the highest yield in *Scenedesmus quadricauda* briefly, the main results are:

1- Urine is a reliable and sustainable alternative to other artificial fertilizer.
2- Increase in concentrations of urine up to 2.0 mg P/L, leads to increase in biomass production.
3- Feeding timing is determinant in to support algae growth and avoids unwanted stresses on algae.
4- Up to 64 mg L^{-1} d^{-1} and total dry weight of 273 mg L^{-1} were achieved in high urine concentration sample (2.2 mg P/L) which is comparable with other studies.
5- Urine consists of high levels of nitrogen in forms of urea, ammonium and nitrate. This level of nitrogen is more than *Scenedesmus quadricauda* can assimilate based on the phosphorus availability, hence nitrogen should be removed in other ways to reach the optimum biomass and also triggering the lipids accumulation by nitrogen removal.

More experiments are needed to determine the optimum urine-based phosphate-P level in culture medium to support the highest biomass yield in the minimum time. TAG production in the nutrients depletion after biomass production should be investigated to identify the appropriate trade-off between urine feeding to produce biomass and reaching the highest lipids production.

CHAPTER 7

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APPENDICES

Experiment 1

Dilution		Return Value Parameters				
Times	Ratio	Value 1	Value 2	Values 3	Average	S.D
1	**1**	9400	8931	8557	8963	422
2	**2**	5219	4812	4607	4879	312
3	**4**	2670	2338	2287	2432	208
4	**8**	1116	1146	1177	1146	31
5	**16**	688	603	589	627	54
6	**32**	334	306	293	311	21
7	**64**	172	156.6	147.8	159	12

Experiment 2

Fluorometer Returned Value	Volume of filtered water (L)	Dilution Ratio	Abs $_{750}$	Abs $_{665}$	Abs $_{665K}$	C_v (µg/L)
8897	0.2	3	0.0062	0.5811	0.5749	**784**
6390	0.2	3	0.0064	0.4129	0.4064	**554**
6390	0.2	2	0.0081	0.6208	0.6127	**557**
3122	0.2	1	0.0101	0.5543	0.5442	**247**
5979	0.2	2	0.0061	0.6310	0.6249	**568**
9839	0.2	3	0.0112	0.7124	0.7013	**956**
8284	0.2	3	0.0410	0.5895	0.5485	**748**
2993	0.2	1	0.0060	0.7005	0.6945	**315**
7337	0.2	2	0.0032	0.8318	0.8286	**753**
4522	0.2	2	0.0035	0.5158	0.5123	**465**

Experiment 3

Abs $_{680}$	Initial Filter Weight (g)	Filter and Algae Weight (g)	Filtered Culture Volume (l)	Dry Mass (g/L)
0.2639	0.0871	0.1085	0.25	0.0856
0.2803	0.0882	0.1088	0.25	0.0824
0.2039	0.088	0.1041	0.25	0.0644
0.2498	0.0871	0.1071	0.25	0.08
0.1860	0.0886	0.104	0.25	0.0616
0.4507	0.0884	0.1211	0.25	0.1308
0.6619	0.0886	0.1319	0.25	0.1732

Chl-a Index	Initial Filter Weight (g)	Filter and Algae Weight (g)	Filtered Culture Volume (l)	Dry Mass (g/L)
3402	0.0871	0.1085	0.25	0.0856
7695	0.0882	0.1088	0.25	0.0824
11502	0.088	0.1041	0.25	0.0644
17172	0.0871	0.1071	0.25	0.08
29160	0.0886	0.104	0.25	0.0616
41958	0.0884	0.1211	0.25	0.1308
54675	0.0886	0.1319	0.25	0.1732

Experiment 4

		Human Urine as Nutrient Source – Fluorometer Values					
Day	**Control**	**U1 0.2 mg P/L**	**U2 0.5 mg P/L**	**U3 0.8 mg P/L**	**U4 1.1 mg P/L**	**U5 1.4 mg P/L**	**U6 1.7 mg P/L**
1	2279	1712	1870	1672	1899	2293	2207
2	4142	6886	8143	7384	5927	4448	6738
3	7263	6957	7874	8088	13376	12556	12090
4	8372	8126	9849	10068	14154	13608	11250
5	8553	8270	7808	7627	12016	12914	10862
6	7440	6799	7611	8027	10128	12548	10496
7	7193	6694	7328	7604	9768	11498	9176
8	7089	5768	6897	6327	9698	11016	8199
9	5653	5880	6515	6615	7637	9800	7626
10	5251	4915	5850	5065	5748	7808	6260
11	4221	4598	5139	4850	5695	7422	5805
12	4229	3896	4309	4036	5109	5596	4845
13	3151	3138	3769	4360	3031	5846	3927

		Chemical Fertilizer as Nutrient Source – Fluorometer Values					
Day	**Control**	**F1 0.2 mg P/L**	**F2 0.5 mg P/L**	**F3 0.8 mg P/L**	**F4 1.1 mg P/L**	**F5 1.4 mg P/L**	**F6 1.7 mg P/L**
1	2279	2106	1872	2160	2123	2129	2433
2	4142	5252	7333	4931	4705	7530	4639
3	7263	6134	6460	8093	8167	8227	7862
4	8372	7164	8388	8461	9750	9793	8835
5	8553	7176	7941	8494		7980	8089
6	7440	6057	6739	7068	6680	6799	7065
7	7193	5835	6646	6080	6385	5442	6554
8	7089	4957	5113	5695	5930	5753	6120
9	5653	3986	5270	5297	4608	4507	4908
10	5251	3093	4367	4494	3870	3845	4422
11	4221	2695	4138	3634	3336	3541	3300
12	4229	2739	3636	3486	3646	3360	3282
13	3151	2412	3420	3526	3320	3205	2738

Experiment 5

Day		Urine Sample – Fluorometer values					
Since phase 1	Since phase 2	U1	U2	U3	U4	U5	U6
13	1	3138	3769	4360	3031	5846	3927
14	2	10952	16132	13730	14628	15020	19252
15	3	10998	16038	13502	13022	15134	17278
16	4	11270	15282	13486	11128	15264	14784
17	5	10466	15134	13076	11006	15978	13782
18	6	10098	14978	11620	10780	14554	12896
19	7	10076	11378	10096	5372	10626	10816
20	8	25914	37292	36540	22008	31285	30800
21	9	31740	42655	40455	23916	41220	35232
22	10	34444	49145	42905	26504	48675	41960
23	11	35370	53010	53874	27968	51216	52808
24	12	34555	48111	44681	24872	42984	41982
25	13	33375	40578	35016	18488	37494	40200
26	14	21376	28484	21356	9378	20324	23444
27	15	36745	48535	50514	34596	41608	52520
28	16	50015	57960	48008	42910	38725	59296
29	17	58320	65555	55480	48083	40968	68008
30	18	55992	70656	60160	55176	57992	70576
31	19	52800	75192	64496	57608	65064	73080
32	20	42735	78099	60123	46263	61887	71127

Experiment 6

	Fluorometer values					
Day	U1	U2	U3	U4	U5	U6
0	2754	1866	3219	2127	3768	3233
1	2608	2579	3880	2027	3846	3289
2	17544	16876	24723	10768	21855	21447
3	18644	17745	36720	12796	28020	30308
4	33820	43520	54828	18888	45605	46155
5	35904	53214	66288	17340	57575	67669
6	37644	72296	75960	21702	56800	71656
7	34254	65604	71408	21279	51072	64582
8	41955	68495	70088	23982	63007	64295
9	46386	60669	66080	26517	58618	66976
10	47105	68704	70808	17768	54600	67970
11	35886	56938	64704	23740	53438	75429
12	31596	57344	66661	29670	64264	92210
13	35610	59984	69888	32715	73870	106692
14	41328	69468	82940	32210	82010	110652
15	50817	82576	136052	34410	85872	117384
16	49621	79660	114807	36486	80136	116865
17	59766	76986	89670	41307	97545	124992

Experiment 7

Grow Rate in Distilled Water								
	DW1		DW2		DW3			
Day	Chl Index	pH	Chl Index	pH	Chl Index	pH	Chl Average	Chl Standard Deviation
0	4410	9.02	2541	9.05	3633	9.03	3528	939
1	3402		3402		3507		3437	60
2	4340	9.11	4092	9.23	5177	8.9	4536	568
3	8990		6975		7905		7957	1008
4	8866	9.79	7781	9.86	8928	9.79	8525	645
5	15469		12989		14973		14477	1312
6	15662	10.09	8405	10.17	10578	10.01	11548	3725
7	24928		19229		17917		21921	5836
8	28495	8.55	24313	8.26	23739	7.89	26336	4011
9	32964		23657		30135		29602	5697
10	37638	10.12	33948	9.85	33046	9.64	36244	4779

Grow Rate in Tap Water								
	TW1		TW2		TW3			
Day	Chl Index	pH	Chl Index	pH	Chl Index	pH	Chl Average	Chl Standard Deviation
0	5082	7.7	4620	7.74	4620	7.73	4774	266
1	3192		3297		2415		2968	481
2	6386	8.93	8091	8.51	5628	8.57	6702	1261
3	15376		13609		12989		13991	1239
4	19561	9.55	18259	9.43	15841	8.8	17887	1888
5	25823		25544		21235		24201	2572
6	23001	10.61	21033	9.77	20131	9.46	21388	1468
7	28003		24969		19468		24147	4327
8	37269	9.57	32595	8.34	30668	8.26	33511	3394
9	33046		35137		32144		33442	1535
10	38581	10.33	37597	9.3	35014	8.96	37064	1842

Experiment 8

Grow Rate without Air Bubbling (NB)										
Day	NB1		NB2		NB3		Chlorophyll		pH	
	Chl Index	pH	Chl Index	pH	Chl Index	pH	Average	S.D.	S.D.	Average
0	2856	8.36	3171	8.44	3083	8.39	3037	163	0.037	7.63
1	6468		6048		6716		6411	338		
2	8757	8.72	8526	8.75	7938	8.71	8407	422	0.019	7.94
3	12957		9345		9597		10633	2017		
4	17535	10.01	16968	9.77	11613	9.16	15372	3268	0.404	8.79
5	17763		16120		23064		18982	3629		
6	22537	10.33	18073	10.2	18476	9.93	19695	2469	0.188	9.25
7	17143		16306		23870		19106	4147		
8	19685	10.43	18724	10.45	27745	10.56	22051	4954	0.065	9.55
9	15949		19721		33210		22960	9075		
10	10660	10.41	16031	10.45	11275	10.52	12655	2940	0.051	9.54
11	15129		23534		23083		20582	4728		
12	16810	10.02	18450	9.78	30627	9.91	21962	7548	0.12	9.90
13	19065		27839		42804		29903	12003		
14	21730	10.18	25912	9.72	31816	10.24	26486	5067	0.284	10.05

Grow Rate under Half-time Air Bubbling (HB)										
Day	HB1		HB2		HB3		Chlorophyll		pH	
	Chl Index	pH	Chl Index	pH	Chl Index	pH	Average	S.D.	S.D.	Average
0	3486	7.68	3345	7.67	3742	7.61	3525	201	0.038	7.65
1	8131		7241		6546		7305	794		
2	9282	8.53	10620	8.62	9660	8.51	9853	689	0.059	8.55
3	12012		13839		13104		12985	919		
4	17073	10.02	15288	9.45	15267	9.19	15876	1036	0.425	9.55
5	28799		24614		26691		26701	2092		
6	31682	10.31	32581	9.89	34007	9.73	32757	1172	0.3	9.98
7	30163		39618		34286		34689	4740		
8	37014	10.39	30690	10.22	41385	10.23	36363	5377	0.095	10.28
9	47027		52480		51619		50375	2931		
10	29971	10.44	35752	10.43	34522	10.44	33415	3045.3	0.006	10.44
11	33415		34973		37515		35301	2069		
12	44485	9.21	56703	9.26	58015	9.51	53068	7461	0.161	9.33
13	36203		46535		52521		45086	8254		
14	42025	9.3	53300	9..00	59614	9.98	51646	8910	0.502	9.43

Grow Rate under Full-time Air Bubbling (FB)										
Day	FB1		FB2		FB3		Chlorophyll		pH	
	Chl Index	pH	Chl Index	pH	Chl Index	pH	Average	S.D.	S.D.	Average
0	3581	8.5	4148	8.44	4166	8.39	3965	333	0.051	7.68
1	7253		6930		6447		6877	405		
2	10424	8.3	9276	8.37	10521	8.4	10074	692	0.047	7.60
3	13104		11634		13818		12852	1113		
4	19131	8.7	15183	8.96	13377	8.18	15897	2942	0.366	7.83
5	25482		28923		25358		26588	2023		
6	24893	9.22	24893	8.82	22103	8.82	23963	1610	0.213	8.15
7	31217		41726		26381		33108	7845		
8	27466	10.15	26288	9.89	28954	8.78	27569	1336	0.671	8.75
9	34973		49241		40180		41465	7220		
10	34276	9.84	31447	10.48	32554	9.33	32759	1425	0.531	9.00
11	24026		29110		37761		30299	6944		
12	46125	8.69	65190	9.55	41410	8.39	50908	12591	0.602	8.88
13	23329		37802		28577		29903	7327		
14	34317	9.48	56539	10.13	28987	8.82	39948	14614	0.655	9.48

Experiment 9

Urine 530 µg/l Series											Chlorophyll index		pH	
Day	**Rep 1**	**pH 1**	**Rep 2**	**pH 2**	**Rep 3**	**pH 3**	**Rep 4**	**pH 4**	**Rep 5**	**pH 5**	**Average**	**S.D.**	**Average**	**S.D.**
1	2780		2780		2780		2780		2780		2780			
2	8181	9.16	8748	9.22	9477	9.14	8667	9.09	9388	9.04	8892	540	9.13	0.07
3	21708	9.66	22680	10.31	22113	10.22	19140	10.48	22680	10.23	21664	1469	10.18	0.31
4	30132	10.57	15795	10.63	16646	10.57	13122	10.59	15390	10.58	18217	6787	10.588	0.03
5	16038	10.71	12312	10.78	13770	10.76	13932	10.74	12231	10.69	13656	1549	10.736	0.04
6	24057	10.57	20007	10.57	15147	10.54	18954	10.55	23328	10.45	20299	3596	10.536	0.05
7	28836	10.69	11421	10.57	13041	10.57	10287	10.58	10611	10.5	14839	7897	10.582	0.07
8	34587	10.61	14094	10.57	13851	10.6	11340	10.63	11178	10.58	17010	9920	10.598	0.02
9	43578	10.61	26163	10.68	18873	10.71	10935	10.72	16767	10.73	23263	12595	10.69	0.05

Bold's Series											Chlorophyll index		pH	
Day	**Rep 1**	**pH 1**	**Rep 2**	**pH 2**	**Rep 3**	**pH 4**	**Rep 4**	**pH 4**	**Rep 5**	**pH 5**	**Average**	**S.D.**	**Average**	**S.D.**
1	3200		3200		3200		3200		3200		3200			
2	4883	6.86	4014	6.64	5847	6.64	4047	6.72	5068	6.76	4772	767	6.724	0.09
3	9801	7.12	6885	6.79	12312	6.86	6075	6.94	9882	7.08	8991	2521	6.958	0.14
4	11340	7.1	8505	6.9	9720	6.87	8424	6.99	10530	7.1	9704	1268	6.992	0.11
5	5751	7.21	5184	6.96	5103	6.95	6480	7.07	5184	7.21	5540	586	7.08	0.13
6	4050	7.34	3726	7.16	2754	7.16	4293	7.26	3159	7.34	3596	634	7.252	0.09
7	2349		3240		1944		3726		2187		2689	759		

Fertilizer Series											Chlorophyll index		pH	
Day	Rep 1	pH 1	Rep 2	pH 2	Rep 3	pH 4	Rep 4	pH 4	Rep 5	pH 5	Average	S.D.	Average	S.D.
1	3314		3314		3314		3314		3314		3314			
2	3618	8.76	2573	8.78	3678	8.73	2219	8.83	2558	8.76	2929	672	8.772	0.04
3	2303	9.2	1435	9.37	1959	9.12	1771	9.34	1436	9.24	1781	368	9.254	0.10
4	567	8.98	243	9.31	486	9.09	810	9.02	381	9.05	497	213	9.09	0.13
5	324	9.04	162	9.65	324	9.39	486	9.48	243	9.51	308	120	9.414	0.23
6	243	9.09	162	10.23	243	9.95	324	10.34	162	10.21	227	68	9.964	0.51
7	405		0		81		81		0		113	168		

Urine 1.8 mg P/L Series											Chlorophyll index		pH	
Day	Rep 1	pH 1	Rep 2	pH 2	Rep 3	pH 3	Rep 4	pH 4	Rep 5	pH 5	Average	S.D.	Average	S.D.
1	3772		3772		3772		3772		3772		3772			
2	6614	8.61	6554	8.66	6038	8.76	6492	8.59	5890	8.65	6318	330	8.654	0.07
3	15066	9.4	20331	9.25	18873	9.48	16848	9.34	19602	9.27	18144	2156	9.348	0.09
4	29241	10.61	35235	10.12	27135	10.48	31185	10.45	31509	10.19	30861	3007	10.37	0.21
5	19845	10.68	26649	10.51	23004	10.49	21303	10.53	27297	10.49	23620	3267	10.54	0.08
6	20493	10.33	29808	10.33	35964	10.05	29403	10.29	29727	10.4	29079	5527	10.28	0.13
7	19845	10.21	35154	10.23	33858	9.99	32076	10.31	40824	10.34	32351	7720	10.216	0.14
8	30132	9.86	56700	10.6	34344	9.92	39933	10.29	47142	10.3	41650	10555	10.194	0.30
9	41229	9.95	54513	9.89	34830	9.2	32238	10.28	41067	10.26	40775	8620	9.916	0.44

Experiment 10

U-2.0 Series : Chlorophyll index							
Day	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**	**Average**	**S.D.**
1	1863	1863	1701	2025	1782	1863	132
2	3564	3159	2754	1620	2835	2774	838
3	5832	6723	5589	2997	4617	5285	1602
4	11193	12960	16362	8667	12555	12295	3233
5	16119	19116	21546	20250	22194	19257	2316
6	22032	29646	31266	30132	27459	28269	4213
7	35235	47547	45846	41877	26730	42626	5470
8	38313	52488	54351	49896	24381	48762	7201
9	34668	54837	57348	44064	13851	47729	10441
10	56052	93808	84645	64800	13527	74826	17413
11	43578	57915	59616	45846	12879	51738	8196
12	36045	53379	69255	43011	-	50422	14434
13	32400	50544	62370	33291	-	44651	14466
14	30345	53321	63789	33677	-	45283	15968
15	29727	62046	71280	34506	-	49389	20392
16	25250	52312	63213	30785	-	42890	17884
17	22761	46170	61074	28674	-	39669	17390

U-2.5 Series : Chlorophyll index							
Day	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**	**Average**	**S.D.**
1	2106	1863	1863	1701	1620	1830	186
2	2268	2511	2916	2835	2511	2608	265
3	4941	7047	6318	6480	5832	6123	791
4	9558	14661	13689	15066	11664	12927	2297
5	15876	19521	17415	17901	18468	17836	1347
6	25110	28593	23652	20574	24948	24575	2891
7	36774	40257	28026	21708	33615	32076	7336
8	48762	47304	31185	23490	39366	38021	10738
9	51516	44064	27135	24705	45765	38637	11964
10	84078	66744	43011	43983	60993	59761	17111
11	55404	43578	31428	28188	45117	40743	11029
12	51678	37017	24381	22437	41472	35397	12185
13	47790	30051	20817	23976	39204	32367	11111
14	57233	32897	24241	28234	47134	37947	13821
15	72333	37422	28350	33048	65286	47287	20062
16	52455	25341	18324	24885	56732	35547	17672
17	35316	16929	14175	19197	47304	26584	14207

U-3.0 Series : Chlorophyll index							
Day	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**	**Average**	**S.D.**
1	1458	1296	1215	1458	1053	1296	172
2	2430	2673	2673	2592	3402	2754	376
3	5427	4779	6399	6804	6399	5961	833
4	11502	15795	13932	14742	12150	13624	1784
5	16767	23166	19521	18792	18468	19342	2364
6	16605	27459	16929	21465	22275	20946	4456
7	17658	35397	20331	23490	24948	24364	6784
8	16767	38475	22761	23085	22842	24786	8100
9	14580	35154	22680	19197	26325	23587	7786
10	16443	43173	29079	24138	18630	26292	10643
11	9558	25920	21222	19278	9720	17139	7261

U-3.5 Series : Chlorophyll index							
Day	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Rep 5**	**Average**	**S.D.**
1	1701	972	1215	891	1134	1256	313
2	1377	2592	2106	3645	3078	2288	726
3	2997	6723	6561	9234	7695	5994	2060
4	12960	17010	14580	17172	13851	14600	1738
5	20088	18954	19926	17496	16605	18893	1606
6	23085	21384	20493	14013	17334	20574	2413
7	27054	21627	19683	9477	18387	21688	3817
8	24057	18630	12798	5994	13203	14936	6789
9	26568	20250	15876	5508	14013	16443	7791
10	22113	12231	10449	4779	7695	11453	6594