# Nutrient recycling:

## Growth of *Chlorella vulgaris* and *Scenedesmus obliquus* on high levels of nutrients and digested pig manure

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## Summary

The Netherlands are dealing with a great overabundance in pig manure, the waste surplus has to be stored or disposed. These are costly matters. A more cost effective purpose could be the commercial growth of microalgae on pig manure, producing algal biomass for use as animal feeds. *Spirulina platensis*, a cyanobacterium, is used in commercial algae production in Asia, but recent studies have showed that it is capable of toxin production. Previous studies indicate that *Chlorella* and *Scenedesmus*, green microalgae, can successfully be grown on pig manure, but it is unknown which of the two species, *Chlorella vulgaris* or *Scenedesmus obliquus*, is more suitable for commercial growth on high levels of pig manure. Therefore, both species were grown on culture media containing 0 – 1000 mg $NH_4^+$ $l^{-1}$, 0 – 500 mg $PO_4^{3-}$ $l^{-1}$, and 0 – 500 mg $K^+$ $l^{-1}$. Furthermore, *C. vulgaris* and *S. obliquus* were grown on dilutions of digested pig manure in groundwater, containing 0 – 50% digested pig manure. The algae were grown for nine days. During this period the chlorophyll-a concentration, the particle concentration, and the biovolume concentration were measured five to six times. The dry weight per liter per treatment was determined after the experiments. The results were compared to the growth of these algae on WC medium, to determine the highest level of ammonium, phosphate, potassium, and digested pig manure at which the yield rate of one of the species was still comparable to the yield rate in WC medium.

At 100 mg $NH_4^+$ $l^{-1}$ and 500 mg $PO_4^{3-}$ $l^{-1}$, the chosen 'highest levels' of ammonium and phosphate, the yield rates based on chlorophyll-a concentration of *C. vulgaris* were significantly higher than the yield rates of *S. obliquus*, but the yield rates of *C. vulgaris* based on particle concentration were significantly lower than the yield rates of *S. obliquus* at 100 mg $NH_4^+$ $l^{-1}$ and 500 mg $PO_4^{3-}$ $l^{-1}$. The differences between the yield rates of *C. vulgaris* and *S. obliquus* based on biovolume concentration at 100 mg $NH_4^+$ $l^{-1}$ and 500 mg $PO_4^{3-}$ $l^{-1}$ were insignificant due to large variations between the replicas. At the 'highest level' of potassium, 500 mg $K^+$ $l^{-1}$, the yield rates based on chlorophyll-a concentration, particle concentration, and biovolume concentration of *S. obliquus* were significantly higher than the yield rates of *C. vulgaris*. However, except from the yield rate ratios based on particle concentration concerning the ammonium and phosphate experiments and the yield rate ratio based on biovolume concentration concerning the potassium experiment, all yield rate ratios of *C. vulgaris* were higher than the ratios of *S. obliquus*.

These results indicate that both *C. vulgaris* and *S. obliquus* can grow on high levels of nutrients, but they do not point out unanimously which algae is more suitable for growth on high levels of nutrients. Yet, both the yield rate and the yield rate ratio based on chlorophyll-a concentration of *C. vulgaris* was significantly higher than the yield rate and yield rate ratio of *S. obliquus* when grown on the dilution containing the 'highest level' of digested pig manure, 2.5% digested pig manure.

# Preface

This research was performed from December 2009 to December 2010 at Aquatic Ecology and Water Quality Management, Department of Environmental Sciences, Wageningen University and Research centre under the supervision of Els Faassen. It was part of the Biology Master at the Radboud University Nijmegen, The Netherlands. The digested pig manure used for this research was provided by Biogreen Salland Heeten (The Netherlands, www.bieleveld.com). The analysis of the copper, zinc, iron, and potassium levels in the digested pig manure and groundwater was performed by the 'Chemisch Biologisch Laboratorium Bodem' (CBLB) of Wageningen University and Research centre. The analysis of nitrogen and phosphate levels in the digested pig manure and groundwater was performed by Wendy Beekman from Aquatic Ecology and Water Quality Management, Department of Environmental Sciences, Wageningen University and Research centre.

## Introduction

The Netherlands are dealing with a great overabundance in pig manure, due intensive livestock farming, and restrictions for using manure as fertilizer on agricultural lands. The regulations were set up to prevent eutrophication and pollution of surrounding land and groundwater. When the limit for manure production is crossed, the waste surplus has to be stored or disposed. These are costly matters, and it would be advantageous for the farmers and the environment if the manure could be used for a different, more cost effective, purpose. An example of such a purpose might be the commercial growth of microalgae on pig manure, producing algal biomass for use as animal feeds. Microalgae have high protein, carbohydrate and vitamin contents (Gantar and Svirčev 2008). *Spirulina platensis*, for instance, is a cyanobacterium which is used for commercial algae production in Asia. This bacterium has great nutritious properties (Vonshak and Richmond 1988), is easy to harvest due to its shape and size (Vonshak 1990), and prefers highly selective environments for growth (Grant, Mwatha et al. 1990). However, cyanobacteria are known for their ability to produce toxic compounds (Gantar and Svirčev 2008). Although *Spirulina* is usually regarded as 'safe', research by Rellán *et al.* (2009) has demonstrated the presence of anatoxin-a in dietary supplements containing this cyanobacterium. Also, several medical cases have been reported in which liver and muscle damages were possibly related to *Spirulina* intake (Iwasa, Yamamoto et al. 2002; Mazokopakis, Karefilakis et al. 2008). Therefore, it might be favourable to use an algal species belonging to the green microalgae, for which no toxin production is known (Walker, Purton et al. 2005). Two green microalgae species commonly used in commercial algal biomass production are: *Chlorella vulgaris* and *Scenedesmus* spp. (Gantar and Svirčev 2008).

*Commercial algae production*

Most mass culturing of microalgae takes place in large open-air systems. This is more economically effective than the use of closed systems, since artificial lighting is needed for optimum growth in closed cultures. However, the growth factors are easier to control in closed systems, and open-air systems are more easily contaminated with other algae and organisms. Therefore, algae species which grow in highly selective environments are often used in these systems. For example, *Chlorella* is often used, because it grows well in nutrient-rich media (Borowitzka 1999).

A commonly used open-air system for mass production is a continuous system, called the raceway pond (figure 1). In these race-way shaped culturing ponds fertilizer and groundwater is often used as medium, and the medium is mixed by means of a paddle-wheel. Another type of open system is the circular pond, in which the medium is agitated by a rotating scraper (Lee 2001).

**Figure 1**. Raceway pond (http://www.algae.wur.nl/)

Addition of $CO_2$ to the medium is important to stimulate algal growth and to prevent high pH values (Sevrin-Reyssac 1998). Also it might be necessary to control the temperature of the medium in order to produce algal biomass all year round. This could be done by placing the pond in a greenhouse (De La Noue and De Pauw 1988).

*Chlorella vulgaris*

*C. vulgaris* is a single cell, coccoid alga of 2.0 to 10.0 µm (figure 2). This green microalga grows well in nutrient-rich media (Soong 1980), and it can be grown under mixotrophic conditions, meaning that it can use carbon dioxide as well as simple organic compounds as carbon sources

(Liang, Sarkany et al. 2009). Furthermore, *C. vulgaris* can grow in media containing high ammonia concentrations (up to 750 – 1000 mg $NH_3$-N $l^{-1}$) although maximum cell densities at these concentrations are significantly lower than in the control cultures and in cultures with lower ammonia concentrations (Tam and Wong 1996). This is interesting, since ammonia and urea levels are generally high in pig manure (Moral, Perez-Murcia et al. 2008; Suresh, Choi et al. 2009), and ammonia toxicity could be a problem in growing algae on raw manure.

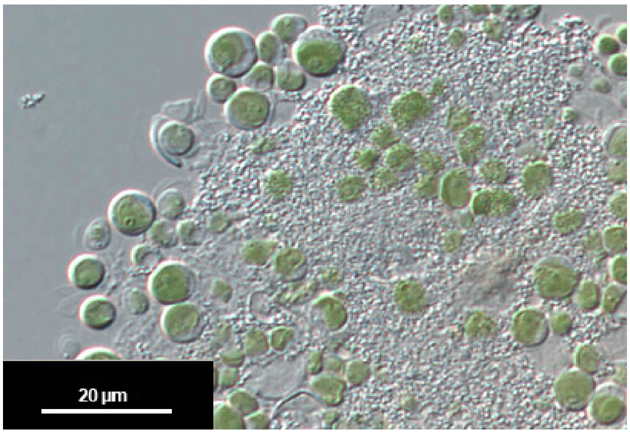


**Figure 2.** *Chlorella vulgaris*. (Laboratoire Génie des Procédés et Matériaux, www.lgpm.ecp.fr)

Dried *Chlorella* biomass contains about 45% protein, 20 % fat, 20% carbohydrate, and 10% minerals and vitamins, making it very suitable as a food supplement for humans and animals (Becker 1994). In addition, *C. vulgaris* produces a broad range of dietary antioxidants, like lutein, astaxanthin, and β-carotene (Vijayavel, Anbuselvam et al. 2007). However, because of *Chlorella's* cell-size, these microalgae are difficult to harvest. A classical way for harvesting small cells is centrifugation, but this requires high investment costs and high energy inputs, causing it to be a very cost-ineffective method. Another harvesting method is flocculation of the algal cells, which makes filtration of the biomass possible. The costs of this method depend on the prize of the flocculant and the amount of flocculant needed to achieve effective flocculation. For use of the algal biomass as food supplement, the flocculant has to be chosen with care, since it should not harm the health of the animals (Vonshak 1990).
A second problem of *Chlorella* is that these microalgae contain a rigid cell wall made of carbohydrates, like cellulose, mannose and xylose. These compounds are indigestible for non-ruminants, therefore the cell wall of the algae has to be ruptured in order to make the *Chlorella* biomass more readily digestible (Gantar and Svirčev 2008).

*Scenedesmus obliquus*

The green microalga *Scenedesmus obliquus* can form regular, two-dimensional aggregates of cells, called coenobia (figure 3). These 'colonies' differ in size and number of cells (Soeder and Hegewald 1992). *S. obliquus* is well adapted to media with high nutrient contents, making it a promising candidate for wastewater treatment (Martínez, Sánchez et al. 2000). An additional advantage of these algae in wastewater treatment is that *Scenedesmus*, like *Chlorella*, is able to grow mixotrophically (Becker 1984). Furthermore, *Scenedesmus* biomass can contain up to 60% crude protein and 14 % lipids. However, another feature *Scenedesmus* has in common with *Chlorella*, is the presence of carbohydrate-rich cell walls, which makes heat-treatment necessary in order to promote the digestibility of the cells (Becker 1984).

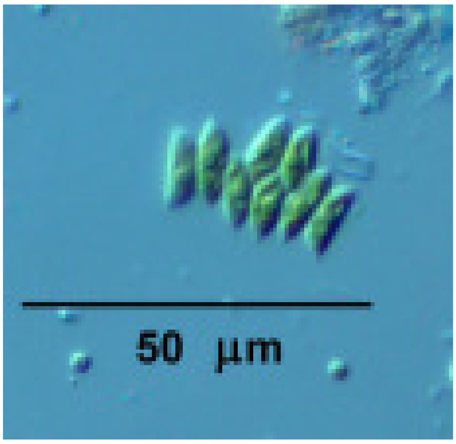


**Figure 3**. Cells of *Scenedesmus obliquus* forming a coenobia. (Phytoplankton and Benthic Algae, http://www.fordham.edu)

Both *C. vulgaris* and *S. obliquus* grow best at a pH of 6.0 – 7.0 (Litchfield 1980), but growth of these algae has been reported at pH values of 5.5 to 9.0 (Greque de Morais and Vieira Costa 2007). The success of algal growth is also determined by the temperature of the medium, and growth of *S. obliquus* has been reported at temperatures between 15 and 35 °C (Hodaifa, Martínez et al. 2010). Maxwell *et al*. (1994) described the growth of *C. vulgaris* at temperatures ranging from 5 to 27 °C. Growth of this alga at higher temperatures has also been reported (Converti, Casazza et al. 2009).

*Algal growth on pig manure*

Concentrations of macronutrients in manure greatly depend on the nutrient intake by the pigs, and excessive levels of these nutrients (mainly N and P) in pig manure are the result of high protein content and indigestible P-containing compounds within pig feed (Aarnink and Verstegen 2007). This leads to very broad ranges of nutrient contents that have been measured in pig manure (table 1). This table shows the 'combined pig slurry' (CPS) values, which means that the values in the table represent combined values of pig slurries from different stages of pig growth. Ammonium concentrations in pig manure are proportional to the urinary urea excretion of pigs (Kerr, Ziemer et al. 2006). When pig manure is stored anaerobically, urea is rapidly converted into ammonium by the enzyme urease, which is present in animal faeces (Béline, Martinez et al. 1998).

The concentrations of heavy metals in manure are a reflection of the heavy metal levels in the feeds consumed by the pigs, and as a consequence heavy metal excretion varies substantially between different stages of pig growth. Especially zinc (Zn)c and copper (Cu) levels can be significant, since these compounds are added to pig feeds to stimulate health (Nicholson, Chambers et al. 1999). Concentrations of Zn and Cu in animal feed differ between countries. For example, Nicholson et al. (1999) found Zn and Cu concentrations in pig feed from the UK which were much higher than the levels of these substances in Dutch pig feeds reported by Jongbloed and Lenis (1993). This is probably the result of tighter restrictions on heavy metal additions in the Netherlands (Nicholson, Chambers et al. 1999). It is known that microalgae accumulate heavy metals (Wilde and Benemann 1992; Yan and Pan 2002), and they can even be used in treatments of waters polluted with heavy metals (Hammouda, Gaber et al. 1995; Travieso, Pellón et al. 2002). In this case, the accumulation of copper and zinc in algal biomass that is subsequently used as animal feed might be beneficial, because heavy metals are removed from the manure, and they serve as health improving minerals in the pig feed.

**Table 1.** Properties and the main nutrient contents of pig manure from South-eastern Spain, South Korea, and the Netherlands. (n.d. = no data)

| | **Southeast Spain[1,2]** | | **South Korea[3]** | | **The Netherlands [4,5]** | |
|---|---|---|---|---|---|---|
| | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** | **Mean** | **St. Dev.** |
| pH | 7.43 | 0.31 | 7.40 | 0.84 | 7.55 | 0.21 |
| Electrical Conductivity (mS $m^{-1}$) | 1790 | 810 | 2710 | 1050 | n.d. | n.d. |
| Total Nitrogen (N) (g $l^{-1}$) | 2.58 | 1.29 | 5.35 | 2.87 | 5.84 | 2.32 |
| Ammoniacal Nitrogen ($NH_3$-N) (g $l^{-1}$) | 2.01 | 1.06 | 3.56 | 2.05 | 3.43 | 1.32 |
| Total Phosphorus (P) (g $l^{-1}$) | 0.76 | 1.04 | 3.38 | 2.57 | 3.68 | 0.97 |
| Potassium (K) (g $l^{-1}$) | 2.26 | 1.27 | 3.08 | 1.54 | 5,89 | 2.25 |
| Copper (Cu) (mg $l^{-1}$) | 42 | 51 | 21 | 26 | n.d. | n.d. |
| Zinc (Zn) (mg $l^{-1}$) | 172 | 176 | 159 | 253 | n.d. | n.d. |

[1] Moral, Perez-Murcia et al. (2005) (Combined Pig Slurry (CPS) values).
[2] Moral, Perez-Murcia et al. (2008) (CPS values).
[3] Suresh, Choi et al. (2009)
[4] http://www.eurolab.nl/meststof-organisch-v.htm. Composition of Dutch pig slurry (combined values of fattener slurry and sow slurry).
[5] Voermans, Van Asseldonk. (1992) Composition of Dutch pig slurry (combined values of fattener slurry and sow slurry).

Manure composition depends on many factors, and it can vary greatly. However, green algae can grow in media with very different nutrient loads. For instance, Gantar et al. (1991) studied the growth of *Spirulina platensis* and *Scenedesmus quadricauda* on various dilutions of liquid pig manure. They found two autochthonous algal species within the manure. One of these species was assumed to be a green alga similar to *Chlorella*, and it was therefore designated as *Chlorella* S. In dilutions containing up to 30% manure, both *Spirulina* and *Scenedesmus* could compete with the autochthonous algae. In dilutions containing higher concentrations of manure algal succession occurred, resulting in the replacement of the introduced algae by the autochthonous algae. Especially *Chlorella* S. was very successful in growing on high concentrations of manure, replacing the introduced *Scenedesmus* and *Spirulina* at 50% manure concentrations after only 8 and 11 days respectively. In addition, growth of *Chlorella vulgaris* on settled and diluted pig manure was described by Travieso *et al*. (2006). They demonstrated that algal growth was stimulated in media containing 250-800 mg Chemical Oxygen Demand per liter (COD $l^{-1}$), but at a COD concentration of 1100 mg $l^{-1}$ growth of *Chlorella* was inhibited. Also, they found that the greatest COD removal efficiency by *Chlorella vulgaris* was at a concentration of 250 mg COD $l^{-1}$ (88% removal), although at concentrations of 400-800 mg COD $l^{-1}$ removal efficiency was still about 60%. Furthermore, Kim et al. (2007) showed the enhanced growth of *Scenedesmus* on fermented pig urine. They have grown a mixed culture of *Scenedesmus* on medium containing 3% fermented pig urine, and compared growth rate, *Scenedesmus* dry weight and nutritious cell content to *Scenedesmus* biomass grown on control medium. All these factors increased significantly, demonstrating the positive effect of culturing *Scenedesmu*s on treated pig urine. Although there was a shortage of inorganic nutrients in the medium, organic materials produced by bacteria during the fermentation process stimulated mixotrophic growth of *Scenedesmus*. Additionally, Sevrin-Reyssac (1998) reported the growth of *Scenedesmus* and *Chlorella* on fermented pig manure. Algal growth on pig manure was successful, but enrichment of the cultures with carbon dioxide, by means of agitation of the medium or by introduction of air enriched in $CO_2$, was necessary to stimulate algal growth and to avoid high pH values.

These previous studies indicate that both *Chlorella* and *Scenedesmus* can successfully be grown on pig manure. However, it is unknown which of the two species, *C. vulgaris* or *S. obliquus*, is more suitable for commercial growth on high levels of pig manure. In order to elucidate which of these to algae species grows best on high levels of pig manure, both species were grown on media containing high levels of ammonia, phosphate and potassium. In addition, *C. vulgaris* and *S. obliquus* were grown on various dilutions of digested pig manure. Growth of the algae was determined by means of measuring the chlorophyll-a concentration, counting the number of particles per milliliter, and measuring the biovolume per milliliter. Also, the dry weight per liter was determined after the experiments.

## Problem definition

*Spirulina platensis*, a cyanobacterium, is used in commercial algae production in Asia, but recent studies have shown that it is capable of toxin production. For development of commercial production of algae on pig manure, it is important to know which non-toxic alga is more suitable for growth on high levels of pig manure: *C. vulgaris* or *S. obliquus*.

## Aim of the Research Project

The aim of this research project was to find out whether *C. vulgaris* and *S. obliquus* are able to grow on pig manure, and to determine which of the two species grows best on high levels of digested pig manure under fixed light, temperature and pH conditions.

## Research questions

*Main research question*

Which of the two selected green microalgae, *C. vulgaris* and *S. obliquus*, is most suitable for commercial algal growth on digested pig manure under the given light, temperature and pH conditions?

*Subquestions and Hypotheses*

To answer the main question, the following subquestions were examined:

- Growth of *C. vulgaris* and *S. obliquus* on high nutrient levels
    - Is the yield rate of *C. vulgaris* or *S. obliquus* higher at high concentrations of ammonium, phoshpate, and potassium? The 'high concentration' is the highest concentration at which the growth rate of one of both algae species is still comparable to or higher than the growth rate of the algae on WC medium.
        - $H_0$: The mean yield rates of *C. vulgaris* and *S. obliquus* are equal when the algae are grown on high concentrations of ammonium, phosphate, and potassium.
        - $H_1$: The mean yield rates of *C. vulgaris* and *S. obliquus* are different when the algae are grown on high concentrations of ammonium, phosphate, and potassium.

- Growth of *C. vulgaris* and *S. obliquus* on Dutch pig manure
    - Is the yield rate of *C. vulgaris* or *S. obliquus* higher at high concentrations of digested pig manure? The 'high concentration' is the highest concentration at which the growth rate of one of both algae species is still comparable to or higher than the growth rate of the algae on WC medium.
        - $H_0$: The mean yield rates of *C. vulgaris* and *S. obliquus* are equal when the algae are grown on high concentrations of digested pig manure.
        - $H_1$: The mean yield rates of *C. vulgaris* and *S. obliquus* are different when the algae are grown on high concentrations of digested pig manure.

## Methods

<u>Growth conditions of the *S. obliquus* and *C. vulgaris* stocks</u>

The algal stocks were grown on modified WC medium (Lurling and Beekman 2006). *Chlorella vulgaris* and *Scenedesmus obliquus* (SAG 276/3A) were grown in batch cultures with volumes of 100 ml, in a Gallenkamp climatic chamber, under continuous light conditions (11.57 µmol $s^{-1}$ $m^{-2}$; standard error 1.12 µmol $s^{-1}$ $m^{-2}$) at 20 °C. The Erlenmeyer flasks were sealed with cellulose plugs, and the batch cultures were not shaken. $HCO_3^-$ was the carbon source within the medium.

The algal stock cultures were transferred to fresh WC medium every two weeks. In addition, the *S. obliquus* and *C. vulgaris* stocks were transferred to fresh WC medium 4-5 days before the start of the growth experiments.

<u>Growth of *C. vulgaris* and *S. obliquus* on high nutrient levels</u>

To study the effect of high nutrient levels on the growth rate of *C. vulgaris* and *S. obliquus*, both species were grown in batch cultures with volumes of 100 ml on WC medium containing various levels of ammonia, phosphate and potassium (table 2).

In addition, *C. vulgaris* and *S. obliquus* were grown in batch cultures with a volume of 100 ml, containing filtered groundwater, and various concentrations of digested pig manure (table 3). Since the filtered pig manure was not sterile, an additional series of the used pig manure dilutions functioned as an extra control to examine potential growth of autochthonous algae. This series was not inoculated with *C. vulgaris* or *S. obliquus*, and was therefore designated as the *No Algae* (NA) series. During all experiments, WC medium was used as a positive control, and all treatments were done in triplicate.

Experimental conditions were similar to the growth conditions described above. Prior to the experiments, the pH of the media containing high levels of ammonium, phosphate, and potassium; and the pH of pig manure dilutions was measured, and adjusted to the pH of WC medium. Subsequently, the electrical conductivity (EC) and salinity of the media and pig manure dilutions were determined with a hand-held probe. The start concentration of algae within the media was 10 µg chlorophyll-a $l^{-1}$ (concentration at t=0).

*S. obliquus* and *C. vulgaris* were allowed to grow over a period of nine days, during this period the chlorophyll-a concentration (µg chlorophyll-a $l^{-1}$), particle concentration (counts $ml^{-1}$), and biovolume concentration ($\mu m^3$ biovolume $ml^{-1}$), were measured 5 or 6 times, with intervals of 1-3 days. For these measurements, subsamples of 10 ml were taken in coulter cups.

The chlorophyll-a concentration was analysed with the PhytoPAM Phytoplankton Analyser (Heinz Waltz GmbH, Effeltrich, Germany). Also, the efficiency of photosystem II (psII-efficiency) was measured in time with the PhytoPAM Phytoplankton Analyser, to evaluate the physiological condition of *C. vulgaris* and *S. obliquus* during the experiments.

The coloration of the digested pig manure and the groundwater caused a bias in the chlorophyll-a concentration measurements with the PhytoPAM Phytoplankton Analyser (Heinz Waltz GmbH, Effeltrich, Germany). Therefore, calibration curves for the chlorophyll-a concentration in different pig manure dilutions were prepared with the PhytoPAM Phytoplankton Analyser, and these were used to correct the chlorophyll-a concentrations measured during the growth experiment.

The number of particles per ml, and the biovolume per ml were determined in duplicate per batch culture, with the CASY® Cell Counter Model TT (Innovatis AG CASY® Technology). The particle volume was calculated by dividing the biovolume per ml at day 9 by the number of particles per ml at day 9. In addition the amount of chlorophyll-a per unit of biovolume, was calculated by dividing the chlorophyll-a concentration at day 9 by the biovolume concentration at day 9.

At the end of each experiment, after nine days of algal growth, the dry weight of the algal biomass was determined by filtration with Whatman GF/C filters as described below.

**Table 2.** Used concentrations of ammonia, phosphate, and potassium during the experiments on growth of *C. vulgaris* and *S. obliquus* on high nutrient levels.

| Experiment | Control | Stock solution ( $NH_4^+$, $PO_4^{3-}$, $K^+$) | $NH_4^+$, $PO_4^{3-}$, $K^+$ Treatments |
|---|---|---|---|
| High levels of ammonia* | WC Medium | 59.307 g $NH_4Cl\ l^{-1}$ | 0, 100, 200, 400, 600, 800 and 1000 mg $NH_3\ l^{-1}$ |
| High levels of phosphate ** | WC Medium | 37.483 g $Na_2HPO_4 \cdot 2\ H_2O\ l^{-1}$ | 0, 25, 50, 200, and 500 mg P $l^{-1}$ |
| High levels of potassium *** | WC Medium | 38.135 g $KCl\ l^{-1}$ | 0, 25, 50, 200, and 500 mg K $l^{-1}$ |

* The WC medium used as a control in the growth experiment on high ammonium levels, also functioned as the medium containing 0 mg $NH_4^+\ l^{-1}$.

** For the growth experiment on high phosphate levels, WC medium (Lurling and Beekman 2006) was used as a control. WC medium was also used as a basis for the media containing 0, 25, 50, 200, and 500 mg $PO_4^{3-}\ l^{-1}$. However, in these media the $K_2HPO_4$ was replaced by KCl.

*** For the growth experiment on high potassium levels, WC medium (Lurling and Beekman 2006) was used as a control. WC medium was also used as a basis for the media containing 0, 25, 50, 200, and 500 mg $K^+\ l^{-1}$. However, in these media the $K_2HPO_4$ was replaced by $Na_2HPO_4 \cdot 2\ H_2O$.

**Table 3.** The manure dilutions that were used for the growth experiment of *S.obliquus* and *C. vulgaris* on digested pig manure. *S. obliquus* and *C. vulgaris* were grown in batches of 100 ml.

| Treatment | Manure/Batch (ml) | Groundwater/Batch (ml) |
|---|---|---|
| 50 % Manure | 50 | 50 |
| 25% Manure | 25 | 75 |
| 5% Manure | 5 | 95 |
| 2.5% Manure | 2.5 | 97.5 |
| Negative Control (groundwater) | 0 | 100 |
| Positive Control (WC Medium) | 0 | 0 |

*Determination of the dry weight by filtration*

Prior to filtration the Whatman GF/C filters (Particle Retention: 1.2 µm) were rinsed with nanopure water, and were dried overnight in aluminium cups in a stove at 103 °C. Subsequently, the filters were allowed to cool down in a dessicator, and each filter was weighted on a Sartorius research balance (Type: R 160 P; Sartorius GmbH, Güttingen, Germany). Then, the contents of the three batches per treatment (ammonium, phosphate, potassium, and digested pig manure) were combined, and 50 ml was taken and filtered over the prepared Whatman filters.

The algal biomass remained on the filter, and after throughout rinsing of the filtration apparatus with nanopure water, the Whatman GF/C filters were dried overnight in aluminium cups at 103 °C**.** Subsequently, the filters were allowed to cool down in a dessicator, and the final weight of each filter with algal biomass was determined with a Sartorius research balance (Type: R 160 P; Sartorius GmbH, Güttingen, Germany). As a control, five extra filters were prepared, and they were washed with 50 ml of nanopure water. The filters were dried and their weight was determined as described above.

After determination of the dry weight of the algal biomass, the increase in biomass per liter per hour was calculated by subtracting the dry weight (mg) of the biomass at the beginning of the growth period from the dry weight (mg) of the biomass at the end of the growth period, multiply the outcome by 20, and divide this by the hours of incubation.

Growth of *C. vulgaris* and *S. obliquus* on digested pig manure

*Pig manure preparation*

The digested pig manure that was used as medium for algal growth was provided by Biogreen Salland (www.bieleveld.com), a company located in Heeten (The Netherlands).

A mixture of pig manure, cereal products and lipids, was digested to produce biogas. Hereafter the digested mixture of pig manure, cereal products, and lipids, is referred to as 'digested pig manure'. The heavier particles within the digested pig manure were removed by means of centrifugation. Hereafter, ultrafiltration was performed to remove the particles that were to light to be removed by centrifugation. Finally, a process called 'reversed osmosis' was used to extract all nitrogen salts and potassium from the digested pig manure, yielding dischargeable water.

For this experiment samples of digested pig manure were taken after ultrafiltration, but prior to reverse osmosis. This means that the used digested pig manure had been centrifuged and filtrated, but was not depleted in nitrogensalts and potassium.

The groundwater which was used to dilute the manure was provided by a pig farm in Zwartbroek (Limburg, the Netherlands). Prior to the growth experiment and measurements, the digested pig manure was filtrated (Whatman GF/C filters) to remove any particles or algae that might have contaminated the non-sterilised digested pig manure in the time between ultrafiltration and the growth experiment. Furthermore, the groundwater was filtered (Whatman GF/C filters) before it was used to dilute the digested pig manure.

*The composition and characteristics of digested pig manure*

The nitrite ($NO_2^-$), nitrate ($NO_3^-$), ammoniacal nitrogen ($NH_3$ – N), and the total nitrogel levels, plus the dissolved phosphorus ($PO_4$ - P) and total phosphorus levels within the digested pig manure where determined with a continuous flow autoanalyser from Skalar (Breda, The Netherlands; www.skalar.com). Five manure samples were taken, and used to produce serial dilutions with nanopure water; yielding five dilutions in which the digested pig manure was 100 times diluted, five dilutions in which the digested pig manure was 1000 times diluted, and five dilutions in which the digested pig manure was 20 000 times diluted. The dilutions were used to measure the nitrite, nitrate, ammonium, total nitrogen, phosphate, and total phosphorus levels as described above.

In addition, 5 samples of undiluted and 10 times diluted groundwater were measured. Prior to this measurement, the groundwater was filtered with a Whatman GF/C filter.

Furthermore, potassium (K), iron (Fe), copper (Cu), and zinc (Zn) levels were determined in digested pig manure and filtered (Whatman GF/C filters) groundwater by means of Inducted Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) by the 'Chemisch Biologisch Laboratorium Bodem' (CBLB) of Wageningen University and Research centre. Five manure samples were taken, and used to produce serial dilutions with nanopure water; yielding five dilutions in which the digested pig manure was 10 times diluted, and five dilutions in which the digested pig manure was 100 times diluted. Levels of potassium, iron, copper, and zinc were measured in these dilutions and in 5 samples of undiluted digested pig manure as described above. Also, five samples undiluted, filtered groundwater were measured.

Yield rate determination

The growth of *Chlorella vulgaris* and *Scenedesmus obliquus* was calculated in four different ways:

1. Based on the chlorophyll-a concentration (µg chlorophyll-a $l^{-1}$).
2. Based on particle concentration (number of particles $ml^{-1}$).
3. Based on biovolume concentration ($\mu m^3$ biovolume $ml^{-1}$).
4. Based on dry weight (mg dry weight $l^{-1}$)

*Calculation of yield rates*

Experimental data were processed in Microsoft Excel. The yield rates during algal growth (Y) were calculated for the proxy measures chlorophyll-a concentration, particle concentration, biovolume concentration, according to the following equation (Wood, Everroad et al. 2005):

$$Y = (N_t - N_0)/\Delta t$$

where $N_0$ is the population size (in µg chlorophyll $l^{-1}$, particles $ml^{-1}$, $\mu m^3$ biovolume $ml^{-1}$) at the beginning of of the growth period, $N_t$ is the population size (in µg chlorophyll-a $l^{-1}$, particles per ml, $\mu m^3$ biovolume $ml^{-1}$) at the end of the growth period, and Δt is the length of the time interval between $N_0$ and $N_t$ (in hours).

After calculation of the yield rates (in µg chlorophyll $l^{-1}$ $h^{-1}$, number of particles $ml^{-1}$ $h^{-1}$, biovolume $ml^{-1}$ $h^{-1}$) per batch, the mean yield rates per treatment were calculated for *C. vulgaris* and *S. obliquus*, and the standard deviation was determined for each treatment.
Since the dry weight was determined for only one sample per treatment, and no replicates were used, it was not possible to calculate mean yield rates and standard deviations.

*Calculation of the yield rate ratios*

The yield rate ratios for *S. obliquus* and *C. vulgaris* were calculated by dividing the yield rates of these algae at the 'highest level' of ammonium, phosphate, potassium, and digested pig manure by their associated yield rate in WC medium. For example, the yield rate of *S. obliquus* at the 'highest level' of ammonium was divided by the yield rate of *S. obliquus* in WC medium that served as positive control during the ammonium experiment.

Statistics

Statistical analysis was performed by using PASW Statistics 18 (SPSS Inc.).

Prior to all statistical tests, two normality tests were carried out (Kolmogorov-Smirnov and Shapiro-Wilk). If the outcome of one of both tests was insignificant, the tested values were considered to be normally distributed.
Prior to the two-way and one-way ANOVA's the homogeneity of variances was tested with a Levene's Test of Equality of Error Variances. If the tested values were not normally distributed or the variances of the tested values were not homogeneous, a series of transformations was performed on the values: log(*value*), log(*value* + 1), log(*value* + 10), 1/log(*value*), √(*value*), *value*$^2$.
Simultaneously with the T-tests, an F-test was performed, to check whether the sample variances were equal.

*Testing the effects of the treatments and algal species on yield rates*

To investigate the effect of both the treatments (ammonium, phosphate, potassium, and digested pig manure) and the algal species (*S. obliquus* and *C. vulgaris*) on the yield rates based on chlorophyll-a concentration, particle concentration, and biovolume concentration, a two-way ANOVA was performed. Each combination of treatment and algal species was given a number (for example: 100 mg $NH_4^+$ $l^{-1}$, *S. obliquus* = 1; 100 mg $NH_4^+$ $l^{-1}$, *C. vulgaris* = 2) in order to be able to conduct a Tukey-HSD test to determine homogeneous groups.
If the tested yield rates were not normally distributed or the variances were not homogeneous, and transformation of the yield rates did not yield normally distributed values and homogeneous variances, two one-way ANOVA's were performed, to test the effect of the treatments (ammonium, phosphate, potassium, and digested pig manure) on the yield rates based on chlorophyll-a concentration, particle concentration, and biovolume concentration of both *S. obliquus* and *C. vulgaris*. Homogeneous groups were determined with a Tukey-HSD test.

Again, if the yield rates were not normally distributed or the variances were not homogeneous, and none of the transformations led to normally distributed values with homogeneous variances, a non-parametric test (Independent Samples Kruskal-Wallis Test) was performed. When the outcome of this test was significant ($p < 0.05$), the yield rates associated with the ammonium, potassium, phosphate, or digested pig manure treatments were compared pairwise per algae per experiment by means of T-tests (for example: the yield rates associated with growth of *S. obliquus* on 100 mg $NH_4^+$ $l^{-1}$ were pairwise compared to the yield rates associated with growth of *S. obliquus* on 200 mg $NH_4^+$ $l^{-1}$, 400 mg $NH_4^+$ $l^{-1}$, 600 mg $NH_4^+$ $l^{-1}$, etcetera). If the yield rates were not normally distributed, they were transformed as described above. When transformation did not yield values that were normally distributed, a nonparametric test (Independent-Samples Mann-Whitney U Test) was carried out.

*Comparison of the yield rates of S. obliquus and C. vulgaris at the 'highest levels' of nutrients and digested pig manure*

The yield rates of *S. obliquus* and *C. vulgaris* at the 'highest levels' of ammonium, phosphate, potassium, and digested pig manure were compared by means of a T-test, to determine whether they differed significantly from each other.

*Testing the effects of the treatments and algal species on particle volume and amount of chlorophyll-a per unit of biovolume*

The effect of the treatments (ammonium, phosphate, potassium) and the algal species (*S. obliquus* and *C. vulgaris*) on the particle volume and the amount of chlorophyll-a per unit of biovolume was examined as described under '*Testing the effects of the treatments and algal species on yield rates*'. The possible differences in particle volumes and amount of chlorophyll-a per biovolume between *S. obliquus* and *C. vulgaris* at highest levels of ammonium, phosphate, potassium, and digested pig manure were analysed as described under '*Comparison of the yield rates of S. obliquus and C. vulgaris at the 'highest levels' of nutrients and digested pig manure*'.

*Comparing the yield rates of S. obliquus and C. vulgaris in WC medium (controls) between experiments*

A one-way ANOVA was performed on the yield rates based on chlorophyll-a concentration, particle concentration, and biovolume concentration of *S. obliquus* and *C. vulgaris* in WC media (controls) , to examine whether these were comparable between the different experiments.

## Results

<u>Yield rates based on chlorophyll-a concentration of *C. vulgaris* and *S. obliquus* after growth on high nutrient levels and dilutions of digested pig manure.</u>

*Ammonium*

High levels of ammonium have a strong effect on the growth of *S. obliquus* and *C. vulgaris*. Even at the lowest concentration that was used, 100 mg $NH_4^+$ per liter, the yield rates of both *S. obliquus* and *C. vulgaris* were lower than the yield rate of these algae in WC medium (figure 4). Also, the growth of *C. vulgaris* in WC medium appeared to be nil in the first two days of the experiment (appendix 1, figure 1.2). However, the chlorophyll-a concentration increased rapidly after the second day, and during the experiment it became clear that, when the cultures were not shaken, *C. vulgaris* tended to stick to the walls of the Erlenmeyer flasks and Coulter cups.

A T-test on the yield rates of *S. obliquus* grown on WC medium, and on WC medium containing 100 mg $NH_4^+$, resulted in a non-significant outcome ($t = 3.80$; $df = 2.01$; $p > 0.05$), suggesting that there was no difference in mean yield rates of S. obliquus when the alga was grown on these media. However, one of the *S. obliquus* cultures grown on WC medium had a yield rate that was very distinct from the other two *S. obliquus* batches on WC medium, resulting in a very large sample variance and standard deviation for the yield rate of this alga on WC medium. Therefore, the T-test was repeated on the logarithm of the yield rates of *S. obliquus*. This led to smaller variances, and showed that there was a significant difference in mean yield rates between WC and WC medium containing 100 mg $NH_4^+$ per liter ($t = 5.25$, $df = 2.08$, $p < 0.05$) (figure 4).

Furthermore, an one-way ANOVA showed that the treatments had significant effect on the mean yield rates of *C. vulgaris* ($F = 29.51$; $df = 6, 14$; $p < 0.01$). The yield rate of *C. vulgaris* in WC medium was significantly different from the yield rates of this algae in the various ammonium treatments (figure 4). Nevertheless, since the mean yield rates in WC medium containing 100 mg $NH_4^+$ per liter were the highest compared to the yield rates in the other media containing $NH_4^+$, 100 mg $NH_4^+$ per liter was taken as the 'highest level' of ammonium.

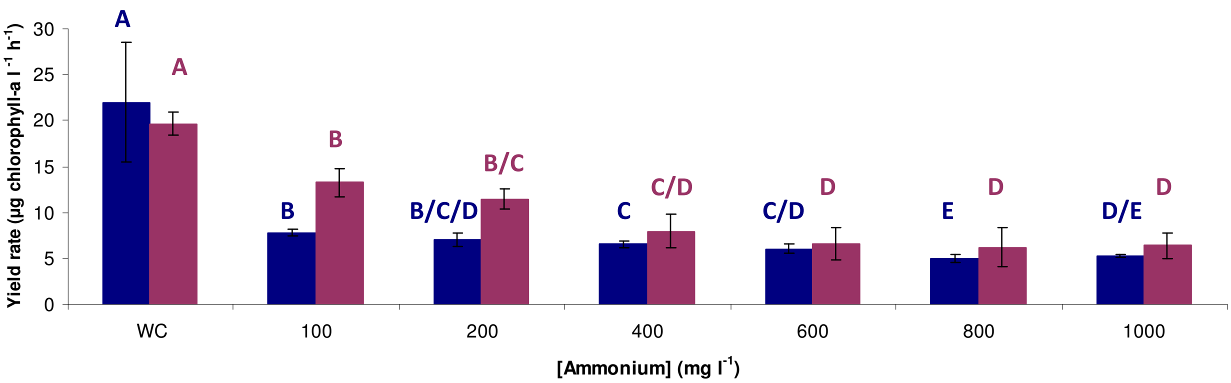


**Figure 4.** Mean yield rates of *S. obliquus* (blue) and *C. vulgaris* (pink) in media containing different levels of ammonium ($NH_4^+$), and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

The mean yield rate of *C. vulgaris* was higher than the mean yield rate of *S. obliquus* at an ammonium level of 100 mg per liter ($t = -6.19$; $df = 2.22$; $p < 0.05$) (figure 4). In addition, the yield rate of *C. vulgaris* at 200 mg $NH_4^+$ $l^{-1}$ was not significantly different from the yield rate of this alga at 100 mg $NH_4^+$ $l^{-1}$. But at higher concentrations of ammonium, the yield rates of *C. vulgaris* were lower. This was also true for *S. obliquus*. In addition, during the growth experiment on high levels of ammonium (100 – 1000 mg $NH_4^+$ $l^{-1}$) the photosystem II efficiency of *C. vulgaris* declined in all ammonium treatments (appendix 2, figure 1.2). Remarkably, the photosystem II efficiency remained comparable to WC medium during the growth experiment on high levels of ammonium (100 – 1000 mg $NH_4^+$ $l^{-1}$) (appendix 2, figure 1.1).

*Phosphate*
In contrast to high levels of ammonium, high levels of phosphate had a positive effect on the mean yield rates of *C. vulgaris*, while the phosphate treatments had a negative effect on the mean yield rates of *S. obliquus* at 500 mg $PO_4^{3-}$ (figure 5). This figure also shows that growth of both algae species was close to zero in the absence of phosphate, indicating the importance of phosphate for algae growth. Furthermore, the photosystem II efficiency of both algae on 0 mg $PO_4^{3-}$ $l^{-1}$ declined during the experiment. The decline in efficiency of *S. obliquus* was gradual from 0.72 to 0.57 (appendix 2, figure 2.1), whereas the efficiency of photosystem II of *C. vulgaris* declined rapidly, from 0.61 at the beginning of the experiment to 0.12 at the end of the experiment (appendix 2, figure 2.2).

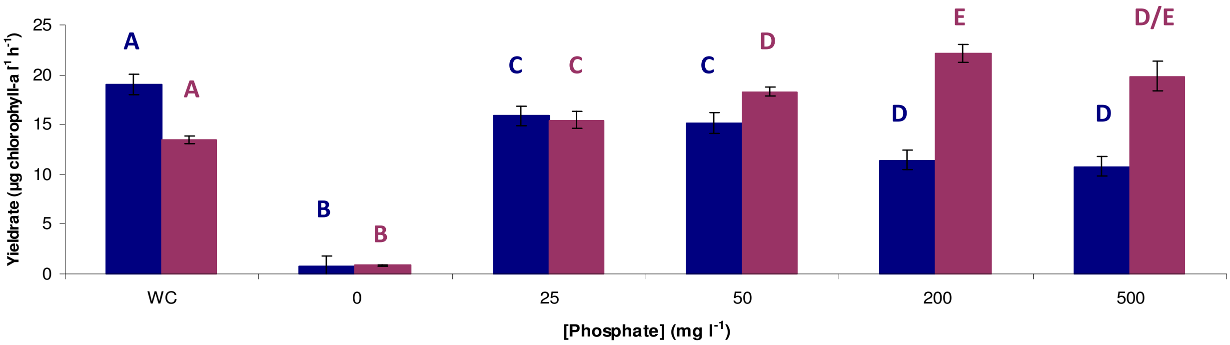


**Figure 5.** Mean yield rates of *S. obliquus* (blue) and *C. vulgaris* (pink) in in WC medium and media containing different levels of phosphate ($PO_4^{3-}$), and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

At the lowest level of phosphate, 25 mg $PO_4^{3-}$ $l^{-1}$, the yield rates af *S. obliquus* and *C. vulgaris* were comparable to each other ($t = 0.78$; $df = 4$; $p > 0.05$). However, at increasing levels of phosphate, the yield rate of *S. obliquus* strongly declined, while the yield rate of *C. vulgaris* was higher than its yield rate on WC medium, even at the highest level of phosphate that was used (500 mg $PO_4^{3-}$ $l^{-1}$, $t = -7.21$; $df = 4$; $p < 0.01$) .
Because the mean yield rate of *C. vulgaris* in 500 mg $PO_4^{3-}$ $l^{-1}$ is higher than the mean yield rate of this alga in WC medium, 500 mg $PO_4^{3-}$ $l^{-1}$ was chosen as the highest level of phosphate at which the mean yield rate of at least one of the algae species was comparable or higher than the mean yield rate in WC medium. However, it was not certain whether the phosphate concentration was really 500 mg $PO_4^{3-}$ $l^{-1}$, since a white precipitation was detected after sterilization of the media containing high levels of phosphate.
Nevertheless, the yield rate of *C. vulgaris* was clearly higher on high levels of phosphate than the mean yield rate of *S. obliquus* at 500 mg $PO_4^{3-}$ $l^{-1}$ ($t = -10.39$; $df = 2.19$; $p < 0.01$).

*Potassium*
The yield rates of *S. obliquus* and *C. vulgaris* after growth on high levels of potassium remained comparable to the yield rates of these algae when grown on WC medium (figure 6).
In the absence of potassium, at 0 mg $K^+$ $l^{-1}$, the yield rates of both algae were significantly lower than their yield rates on WC medium. Also the photosystem II efficiencies of both *S. obliquus* and *C. vulgaris* decreased. The efficiency of photosystem II declined from 0.74 to 0.60 for *S. obliquus* (appendix 2, figure 3.1), and from 0.54 to 0.39 for *C. vulgaris* (appendix 2, figure 3.2).

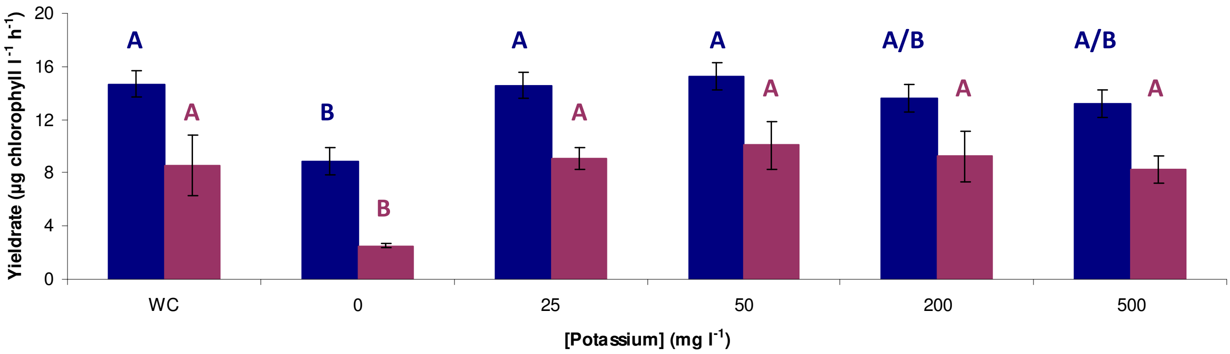


**Figure 6.** Mean yield rates of *S. obliquus* (blue) and *C. vulgaris* (pink) in WC medium and media containing different levels of potassium ($K^+$), and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

At 500 mg $K^+$ $l^{-1}$, the yield rates of both algae were not significantly different from the yield rates when grown on WC medium, therefore, 500 mg $K^+$ $l^{-1}$ was chosen as the highest level of potassium at which the mean yield rate of at least one of the algae species was comparable or higher than the mean yield rate in WC medium.

The mean yield rate of *S. obliquus* at 500 mg $K^+$ $l^{-1}$ was higher than the mean yield rate of *C. vulgaris* at the same potassium level ($t = 5.47$; $df = 4$; $p < 0.01$).

*Manure*

*C. vulgaris* formed particles during the growth experiment on 2.5% and 5% manure, which were visible with the naked eye. Mean yield rates based on chlorophyll-a concentration of the No Algae series were nil or zero (figure 7).

The results indicated that *C. vulgaris* can grow on 100% groundwater. The yield rate of *S. obliquus* at 100% groundwater was nil, and measurements showed that the efficiency of photosystem II declined during the experiment (appendix 2; figure 4.1). The yield rate of *S. obliquus* on 2.5% digested pig manure was significantly lower than on WC medium ($t = 26.58$; $df = 3$; p , 0.01). In addition, the yield rate of *C. vulgaris* on 2.5% digested pig manure was significantly lower than its yield rate on WC medium ($F = 128.82$; $df = 3, 7$; $p < 0.01$) (figure 7).

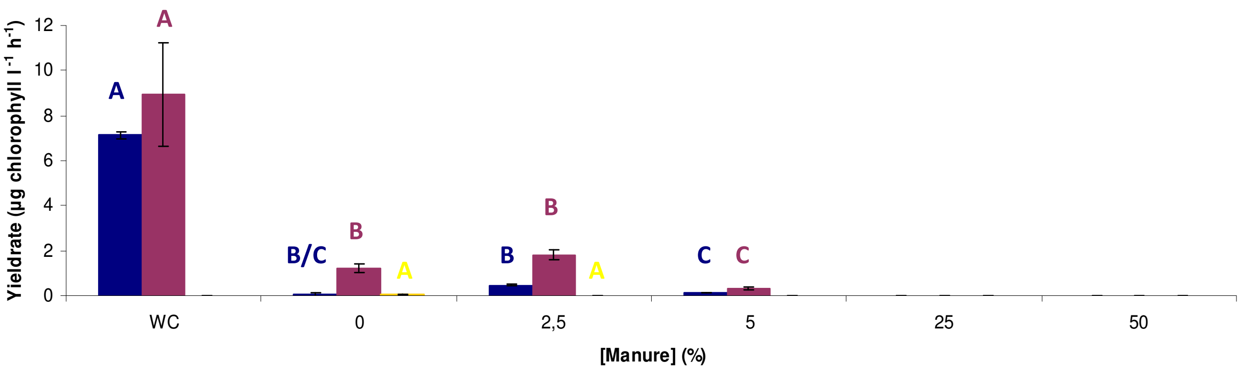


**Figure 7.** Mean yield rates of *S. obliquus* (blue), *C. vulgaris* (pink), and No Algae (yellow) in WC medium and in various dilutions of digested pig manure, and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris,* and yellow capital letters represent the homogeneous subsets of yield rates of the No Algae series..

In spite of this, the mean yield rates in 2.5% digested pig manure were the highest compared to the yield rates in the other media containing digested pig manure, and therefore, 2.5% digested pig manure was selected as the 'highest level of manure'.
The mean yield rate of *C. vulgaris* was significantly higher than the mean yield rate of S. *obliquus*, when the algae were grown on 2.5% digested pig manure ($t = -9.30$; $df = 4$; $p < 0.01$).

<u>Yield rates based on particle concentration and biovolume concentration of *C. vulgaris* and *S. obliquus* after growth on high nutrient levels and dilutions of digested pig manure.</u>

*Ammonium*
In contrast to the yield rates based on chlorophyll-a concentration, the yield rates of *S. obliquus* based on particle concentration were higher than the yield rates of *C. vulgaris*. However, like the yield rates based on chlorophyll-a concentration, the yield rates based on particle concentration of both algae were negativily effected by high concentrations of ammonium (100 – 1000 mg $NH_4^+$ $l^{-1}$) (figure 8).
At the chosen 'highest level of ammonium, 100 mg $NH_4^+$ $l^{-1}$, the yield rate of *S. obliquus* based on particle concentration was significantly higher than the yield rate of *C. vulgaris* ($t = 14.36$; $df = 4$; $p < 0.01$). Furthermore, the yield rates of *S. obliquus* at 100, 200, and 400 mg $NH_4^+$ $l^{-1}$ were comparable ($p > 0.05$), in contrast to the yield rates of this algae based on chlorophyll-a concentration, those differed significantly at 100 and 400 mg $NH_4^+$ ($t = 4.35$; $df = 4$; $p < 0.05$). The yield rates based on particle concentration of *C. vulgaris* at 100 and 200 mg $NH_4^+$ $l^{-1}$ were comparable ($t = 2.06$; $df = 4$; $p > 0.05$), as was the case for this alga's yield rates based on chlorophyll-a. However, the yield rates of *C. vulgaris* at 200 and 400 mg $NH_4^+$ $l^{-1}$ were significantly different ($t = 3.69$; $df = 4$; $p < 0.05$), where as the yield rates of this alga based on chlorophyll-a concentration were comparable at these levels of ammonium.
The yield rates of *S. obliquus* based on biovolume concentration at 200, 400, 600, and 800 mg $NH_4^+$ $l^{-1}$ were comparable to the yield rate of this algae on WC medium ($p > 0.05$) (figure 9). Furthermore, no significant effect of high ammonium levels (100 – 1000 mg $NH_4^+$ $l^{-1}$) on the yield rates based on biovolume concentrations of *C. vulgaris* were detected ($F = 0.53$; $df = 6, 14$; $p > 0.05$) (figure 9). Also, at 100 mg $NH_4^+$ $l^{-1}$, the yield rate based on biovolume concentration of *S. obliquus* is not significantly different from the yield rate of *C. vulgaris* ($t = 0.77$; $df = 4$; $p > 0.05$).

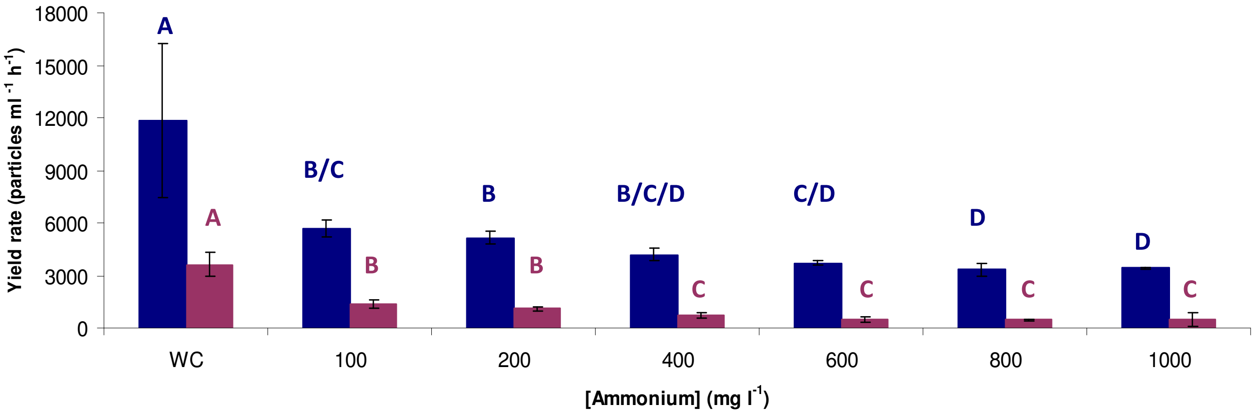


**Figure 8.** Mean yield rates of *S. obliquus* (blue) and *C. vulgaris* (pink) in media containing different levels of ammonium ($NH_4^+$) based on particle concentration, and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

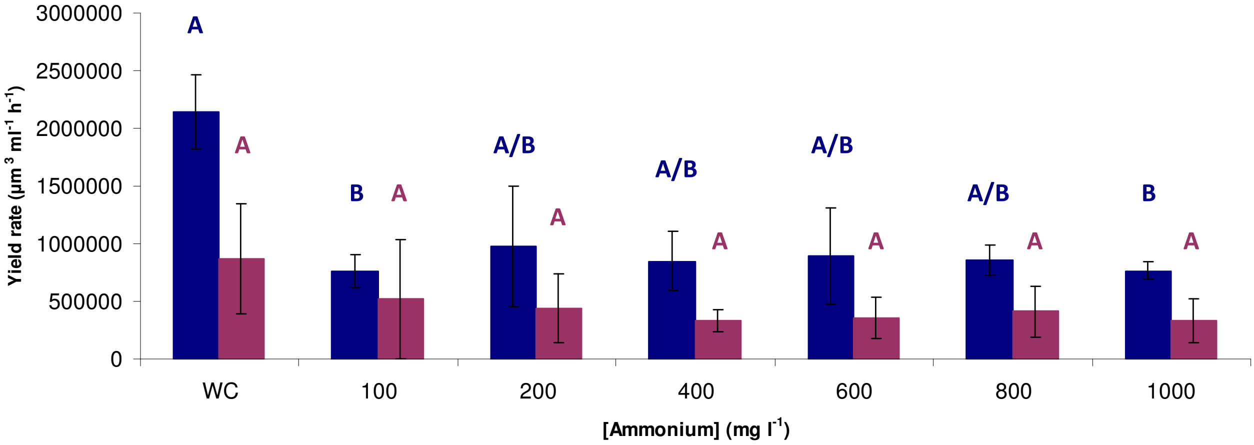


**Figure 9.** Mean yield rates of *S. obliquus* (blue) and *C. vulgaris* (pink) in media containing different levels of ammonium ($NH_4^+$) based on biovolume concentration, and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

*Phosphate*

Like the yield rates based on particle concentration concerning the growth experiment of *S. obliquus* and *C. vulgaris* on high levels of ammonium, the yield rates based on particle concentration of *S. obliquus* concerning the growth experiment on high levels of phosphate were higher than the yield rates of *C. vulgaris* (figure 10. At the highest level of phosphate (500 mg $PO_4^{3-}$ $l^{-1}$) the yield rate of *S. obliquus* was significantly higher than the yield rate of *C. vulgaris* ($t = 16.27$; $df = 4$; $p < 0.01$), in contrast to the results based on chlorophyll-a concentration.

Furthermore, the yield rates based on particle concentration of both algae were negatively influenced by high levels of phosphate (200 – 500 mg $PO_4^{3-}$ $l^{-1}$), whereas the yield rates of *C. vulgaris* based on chlorophyll-a showed a positive response to these phosphate levels when compared to growth of this alga on WC medium.

When phosphate was absent in the medium (0 mg $PO_4^{3-}$ $l^{-1}$) the yield rates of bot algae were low, similar to the results based on chlorophyll-a concentration. The yield rate based on biovolume concentration of *S. obliquus* at 0 mg $PO_4^{3-}$ $l^{-1}$ was significantly different from this alga's yield rate on WC medium ($p < 0.05$), but it was comparable to the yield rates at 50 and 500 mg $PO_4^{3-}$ $l^{-1}$ ($p > 0.05$) (figure 11). Furthermore, the mean yield rate based on biovolume concentration of *C. vulgaris* at 500 mg $PO_4^{3-}$ $l^{-1}$ was higher than its mean yield rate in WC medium, but this difference was not significant ($p > 0.05$). Also, at this phosphate level, the yield rate based on biovolume concentration of *C. vulgaris* was not significantly higher than the yield rate of *S. obliquus* ($t = -2.04$; $df = 4$; $p > 0.05$).

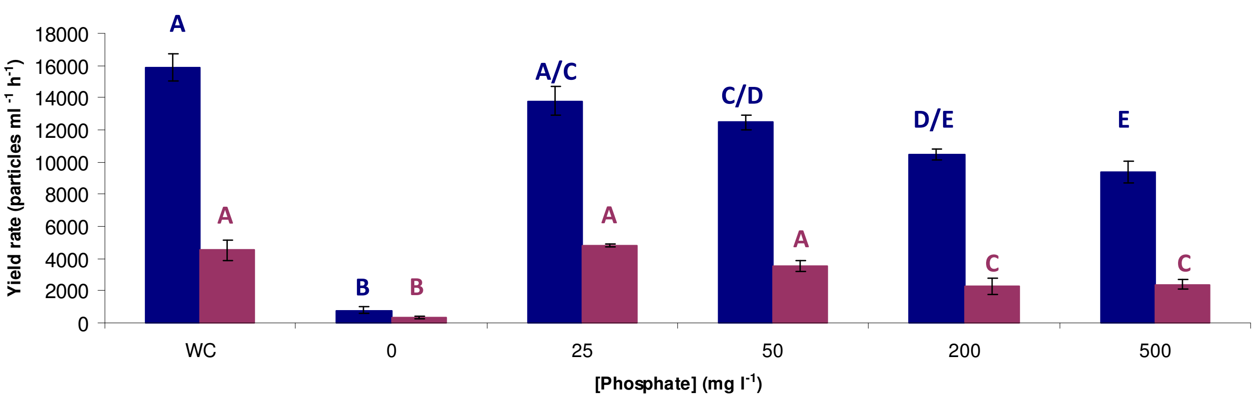


**Figure 10.** Mean yield rates of *S. obliquus* (blue) and *C. vulgaris* (pink) in media containing different levels of phosphate ($PO_4^{3-}$) based on particle concentration, and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

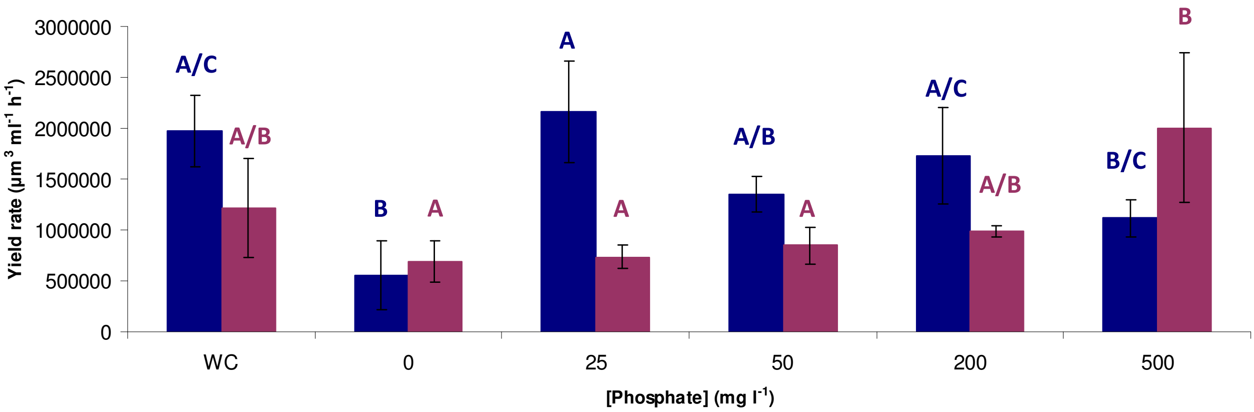


**Figure 11.** Mean yield rates of *S. obliquus* (blue) and *C. vulgaris* (pink) in media containing different levels of phosphate ($PO_4^{3-}$) based on biovolume concentration, and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

*Potassium*

The yield rates based on particle concentration of *S. obliquus* were higher than the yield rates of *C. vulgaris* when these algae were grown on high levels of potassium (0 – 500 mg $K^+$ $l^{-1}$) (figure 12). When potassium was absent, the yield rate based on particle concentration of *S. obliquus* decreased compared to its yield rate on WC medium. On the other hand, the yield rate based on particle concentration of *C. vulgaris* was not significantly different from its yield rate on WC medium when grown on medium without potassium ($p > 0.05$). At 25 – 500 mg $K^+$ $l^{-1}$ the yield rates based on particle concentration were comparable or higher than the yield rates on WC medium for both algae. At the highest level of potassium, 500 mg $K^+$ $l^{-1}$, the yield rate of *S. obliquus* based on particle concentration was sinificantly higher than the yield rate of *C. vulgaris* ($t = 7.19$; $df = 4$; $p < 0.01$).
The various potassium treatments did not affect the yield rates based on biovolume concentration of *S. obliquus* ($F = 0.66$; $df = 5, 12$; $p > 0.05$) and *C. vulgaris* ($F = 1.97$; $df = 5, 12$; $p > 0.05$) (figure 13). Yet, at 500 mg $K^+$ $l^{-1}$, the yield rate based on biovolume of *S. obliquus* was significantly higher than the yield rate of *C. vulgaris* ($t = 6.12$; $df = 4$; $p < 0.01$).

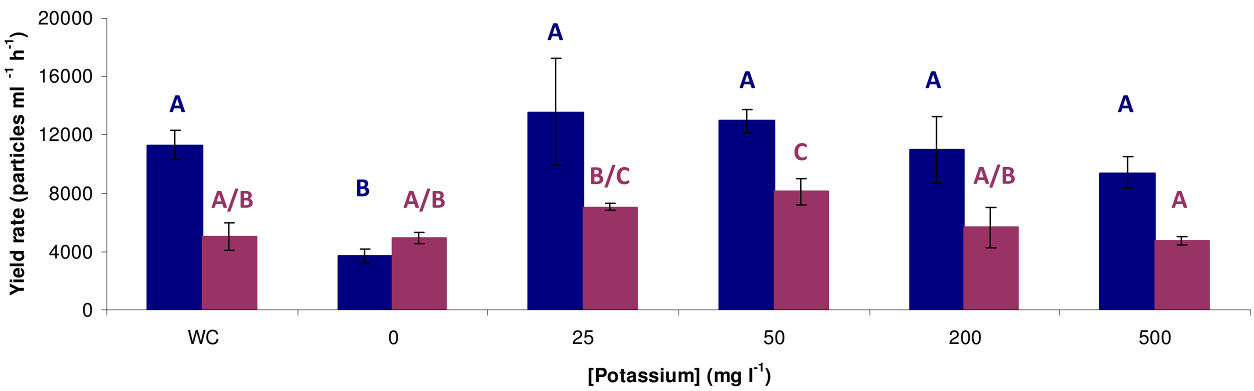


**Figure 12.** Mean yield rates of *S. obliquus* (blue) and *C. vulgaris* (pink) in media containing different levels of potassium ($K^+$) based on particle concentration, and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

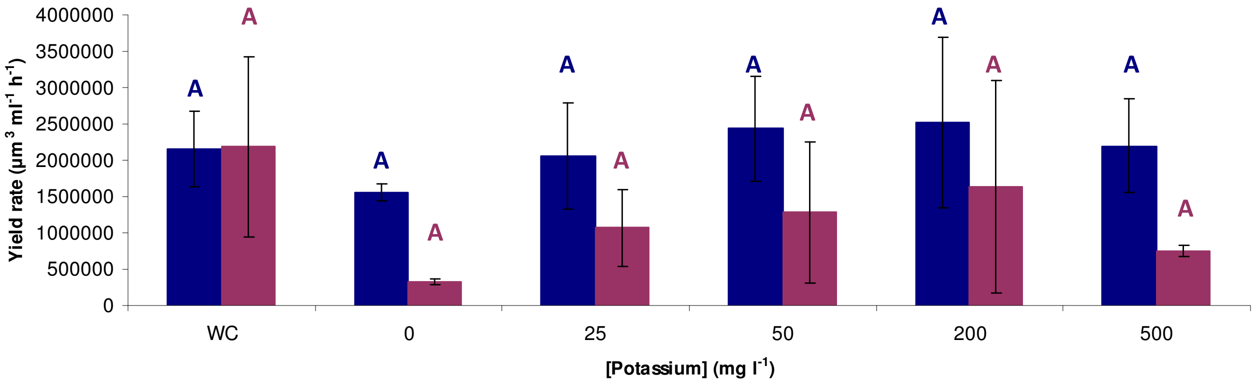


**Figure 13.** Mean yield rates of *S. obliquus* (blue) and *C. vulgaris* (pink) in media containing different levels of potassium ($K^+$) based on biovolume concentration, and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

*Manure*

The yield rates based on particle concentration and biovolume concentration of *S. obliquus* and *C. vulgaris* on digested pig manure were biased by bacterial growth in the unsterilised manure media. Growth of bacterial cells was detected after measurements of particle concentration and biovolume concentration in the No Algae series, and was confirmed by microscopic analysis of the samples. Because the degree of bacterial growth in the series containing *S. obliquus* and *C. vulgaris* was not determined, the yield rates based on particle concentration and biovolume concentration concerning the growth on digested pig manure will not be used for analysis.

<u>Particle volumes of *C. vulgaris* and *S. obliquus* after growth on high nutrient levels.</u>

*Ammonium*

Although the mean particle volumes of *C. vulgaris* increased at high levels of ammonium (100 – 1000 mg $NH_4^+$ $l^{-1}$), a significant effect of the ammonium treatments on the particle volumes of *S. obliquus* and *C. vulgaris* could not be detected (*S. obliquus*: F = 1.17; df = 6, 13; p > 0.05, *C. vulgaris*: F = 1.82; df = 6, 14; p > 0.05) (figure 14). Furthermore, only at 400 mg $NH_4^+$ $l^{-1}$ there was a significant difference between the particle volumes of *S. obliquus* and *C. vulgaris* (t = -4.06, df = 4, p < 0.05).

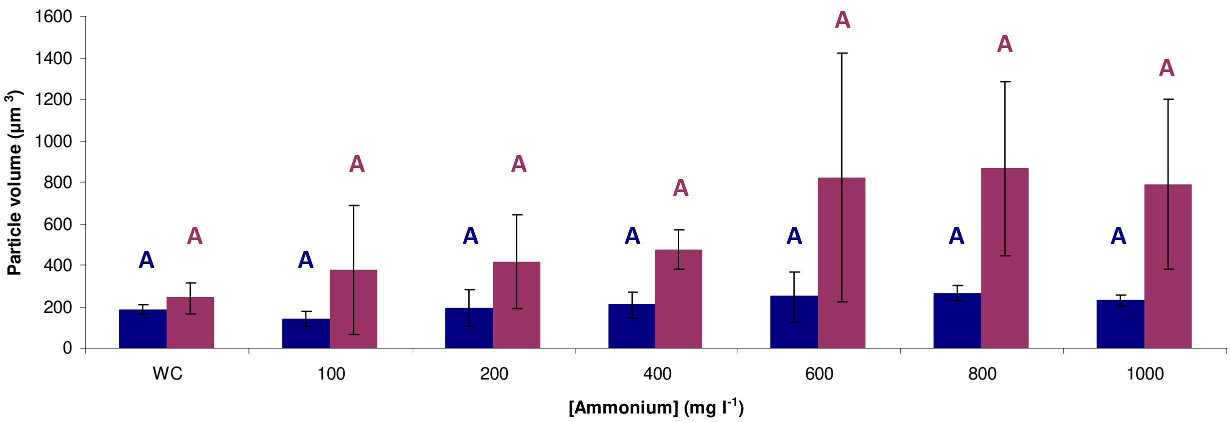


**Figure 14.** Mean particle volumes of *S. obliquus* (blue) and *C. vulgaris* (pink) in media containing different levels of ammonium ($NH_4^+$) on day 9 of the experiment, and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

*Phosphate*

In contrast to the ammonium treatments, the phosphate treatments did have a significant effect on the particle volume of both *S. obliquus* and *C. vulgaris* (*S. obliquus*: $F = 11.63$; $df = 5, 12$; $p < 0.01$, *C. vulgaris*: $F = 32.22$; $df = 5, 12$; $p < 0.01$). In the absence of phosphate (0 mg $PO_4^{3-}$ $l^{-1}$) the particle volumes of both *S. obliquus* and *C. vulgaris* were larger than the particle volumes of these algae in WC medium (figure 15). The particle volumes of *S. obliquus* at high levels of phosphate (25 – 500 mg $PO_4^{3-}$ $l^{-1}$) were comparable to the particle volume of this alga in WC medium ($p > 0.05$). In addition, the particle volumes of *C. vulgaris* at 25 – 200 mg $PO_4^{3-}$ $l^{-1}$ were also comparable to the particle volume of this alga in WC medium. However, at 500 mg $PO_4^{3-}$ $l^{-1}$, the particle volume of *C. vulgaris* was significantly larger than its particle volume in WC medium (figure 15). Furthermore, at 50 – 500 mg $PO_4^{3-}$ $l^{-1}$, the particle volumes of *C. vulgaris* were significantly larger than the particle volumes of *S. obliquus* (50 mg $PO_4^{3-}$ $l^{-1}$: $t = -6.49$; $df = 4$; $p < 0.01$, 200 mg $PO_4^{3-}$ $l^{-1}$: $t = -6.44$, $df = 4$, $p < 0.01$, 500 mg $PO_4^{3-}$ $l^{-1}$: $t = -3.804$; $df = 4$; $p < 0.05$).

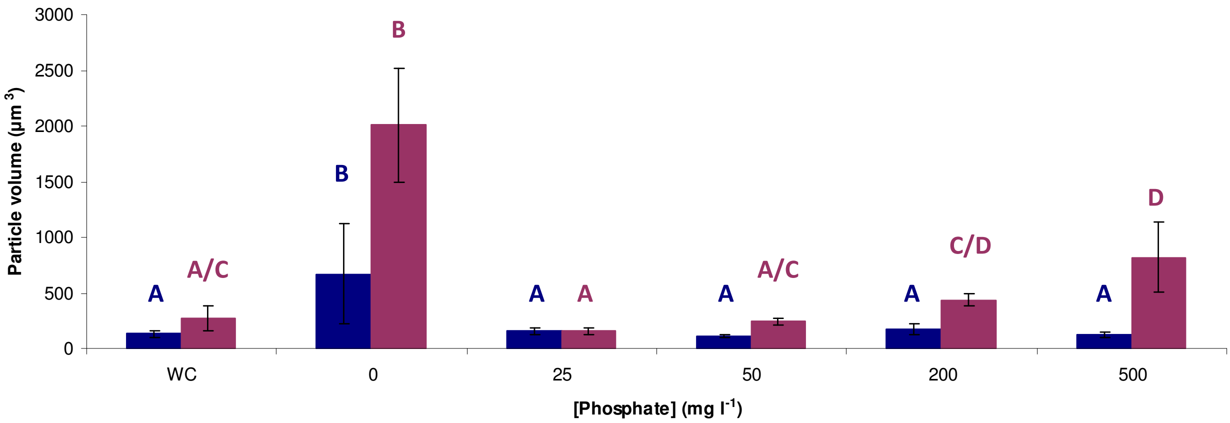


**Figure 15.** Mean particle volumes of *S. obliquus* (blue) and *C. vulgaris* (pink) in media containing different levels of phosphate ($PO_4^{3-}$) on day 9 of the experiment, and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

*Potassium*

Like the posphate treatments, the potassium treatments had a significant effect on the particle volume of both *S. obliquus* and *C. vulgaris* (*S. obliquus*: $F = 9.66$; $df = 5, 12$; $p < 0.01$, *C. vulgaris*: $F = 2.76$; $df = 5, 12$; $p < 0.01$). When potassium was absent (0 mg $K^+$ $l^{-1}$) the particle volume of *S. obliquus* and was larger than its particle volume in WC medium (figure 16). On the other hand, the particle volume of *C. vulgaris* at 0 mg $K^+$ $l^{-1}$ was smaller than the particle volume of this alga in WC medium. At high levels of potassium (25 – 500 mg $K^+$ $l^{-1}$) the particle volumes of both algae were comparable to their particle volumes in WC medium ($p > 0.05$). The particle volume of *S. obliquus* is significanlty larger than the particle volume of *C. vulgaris* at 0 mg $K^+$ $l^{-1}$ and 500 mg $K^+$ $l^{-1}$ (0 mg $K^+$ $l^-$: $t = 10.76$; $df = 4$; $p < 0.01$, 500 mg $K^+$ $l^{-1}$: $t = 2.828$; $df = 4$; $p < 0.05$).

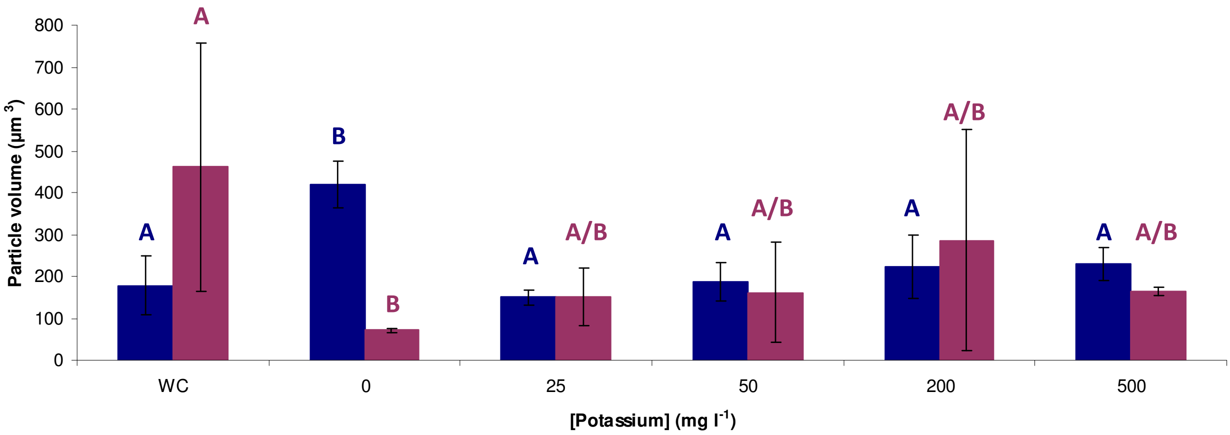


**Figure 16.** Mean particle volumes of *S. obliquus* (blue) and *C. vulgaris* (pink) in media containing different levels of potassium ($K^+$) on day 9 of the experiment, and their standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous subsets of yield rates of *C. vulgaris*.

<u>Comparison within and between experiments based on the ratios of yield rates.</u>

Although algal growth at different nutrient levels can be examined by means of calculating the mean yield rates based on chlorophyll-a concentration, particle concentration, and biovolume concentration; the yield rates based on dry weight (mg $l^{-1}$ $h^{-1}$) were also determined. Table 4 shows the ratios of the mean yield rates of both *S. obliquus* and *C. vulgaris* in media containing 100 mg $NH_4^+$ $l^{-1}$, 500 mg $PO_4^{3-}$ $l^{-1}$, and 500 mg $K^+$ $l^{-1}$, compared to the mean yield rates of these algae in WC medium. Ratios of the yield rates based on chlorophyll-a concentration, dry weight, particle concentration, and biovolume concentration are displayed. Furthermore, table 4 also shows the ratios of the mean yield rates of *S. obliquus* and *C. vulgaris* in dilutions containing 2.5% digested pig manure, compared to the mean yield rate of these algae in WC medium. Only the ratios of the yield rates based on chlorophyll-a concentration, are displayed for the 2.5% digested pig manure dilution (table 4). The ratios based on particle concentration, biovolume concentration, and dry weight, were not used since the results for these variables were biased by bacterial growth in the unsterilised manure media, and the degree of bacterial growth in the series containing *S. obliquus* and *C. vulgaris* was not determined.

The chlorophyll-a concentration yield rates of *S. obliquus* and *C. vulgaris* in WC medium were not similar between experiments (*S. obliquus*: $p < 0.05$; *C .vulgaris*: $F= 26.84$, df = 3, 8; $p < 0.01$), neither were the yield rates in WC medium based on particle concentration (*S. obliquus*: $F = 11.89$; df = 3, 8; $p < 0.01$, *C. vulgaris*: $F = 10.91$, df = 3, 8; $p < 0.01$). The yield rates in WC medium based on biovolume concentration of *S. obliquus* were not similar between experiments ($F = 9.04$; df = 3, 8; $p < 0.01$), whereas the yield rates in WC medium based on biovolume concentration of *C. vulgaris* were similar between experiments ($F = 2.98$; df = 3,8; $p > 0.05$). Still, the yield rate ratios were calculated per experiment, so ratios between and within experiments could be compared.

**Table 4**. The ratios of the mean yield rates of *S. obliquus* and *C. vulgaris* in media containing 100 mg $NH_4^+$ $l^{-1}$, 500 mg $PO_4^{3-}$ $l^{-1}$, and 500 mg $K^+$ $l^{-1}$, compared to the mean yield rate of these algae in WC medium. Plus the ratios of the mean yield rates of *S . obliquus* and *C. vulgaris* in dilutions containing 2.5% digested pig manure, compared to the mean yield rates of these algae in WC medium. The ratios of the mean yield rates in chl-a concentration (µg chlorophyll-a $l^{-1}$ $h^{-1}$), dry weight (mg $l^{-1}$ $h^{-1}$), particle concentration (particles $ml^{-1}$ $h^{-1}$), and biovolume concentration ($µm^3$ $ml^{-1}$ $h^{-1}$) are displayed. (n.a. = not available)

| | | Ratio yield rate chl-a concentration | | Ratio yield rate dry weight | | Ratio yield rate particle concentration | | Ratio yield rate biovolume concentration | |
|---|---|---|---|---|---|---|---|---|---|
| Nutrient | Highest level | S. obliquus | C. vulgaris | S. obliquus | C. vulgaris | S. obliquus | C. vulgaris | S. obliquus | C. vulgaris |
| $NH_4^+$ | 100 mg $NH_4^+$ $l^{-1}$ | 0.35 | 0.67 | 0.28 | 0.51 | 0.48 | 0.39 | 0.46 | 0.51 |
| $PO_4^{3-}$ | 500 mg $PO_4^{3-}$ $l^{-1}$ | 0.57 | 1.47 | 0.62 | 0.98 | 0.59 | 0.52 | 0.56 | 1.65 |
| $K^+$ | 500 mg $K^+$ $l^{-1}$ | 0.90 | 0.96 | 0.83 | 1.00 | 0.83 | 0.95 | 1.02 | 0.34 |
| Manure | 2.5% manure | 0.12 | 0.32 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |

*Ammonium*

The yield rate ratios based on particle concentration and biovolume concentration of *S. obliquus* concerning the ammonium experiment were similar, while the ratios based on chlorophyll-a concentration and dry weight were lower (table 4). Yet, the amount of chlorophyll-a per $µm^3$ of *S. obliquus* at 100 mg $NH_4^+$ $l^{-1}$ was comparable to the amount of chlorophyll-a per $µm^3$ of this algae in WC medium (appendix 3, figure 1).

In contrast to the ratios based on particle concentration and biovolume concentration of *S. obliquus*, the yield rate ratios based on these two variables were not similar for *C. vulgaris*. Instead, the ratios based on dry weight and biovolume concentration of this alga were the same, while its yield rate ratio based on particle concentration was lower. Yet, apart from apart from the ratio based on particle concentration the yield rate ratios of *C. vulgaris* are higher than the ratios of *S. obliquus* for the ammonium experiment.

*Phosphate*

The yield rate ratios of *S. obliquus* were similar between the different measurements within phosphate experiment (table 4), they range from 0.56 based on biovolume concentration, to 0.62 based on dry weight.

On the other hand, the ratios of *C. vulgaris* differ greatly between the methods of measuring within the phosphate experiment. The yield rate ratios based on chlorophyll-a concentration and biovolume concentration are roughly three times as high as the yield rate ratio based on particle concentration. The yield rate based on particle concentration was significanlty lower when *C. vulgaris* was grown on 500 mg $PO_4^{3-}$ $l^{-1}$ than this algas yield rate on WC medium (figure 10), while the particle volume of *C. vulgaris* after growth on 500 mg $PO_4^{3-}$ $l^{-1}$ was significantly larger than its particle volume after growth on WC medium (figure 15). Thus, growth on 500 mg $PO_4^{3-}$ $l^{-1}$ led to less, but bigger particles. Hence, the particle concentration declined, resulting in lower yield rate ratios for this variable.

The ratio of *C. vulgaris* based on chlorophyll-a was almost 1.5 times the ratio based on dry weight. Still, the amount of chlorophyll-a per $µm^3$ of *C. vulgaris* at 500 mg $PO_4^{3-}$ $l^{-1}$ was comparable to the amount of chlorophyll per $µm^3$ of this alga in WC medium (appendix 3, figure 2). Furthermore, although the mean yield rate based on biovolume concentration of *C. vulgaris* at 500 $PO_4^{3-}$ $l^{-1}$ was higher than its mean yield rate in WC medium, the difference was not significant ($p > 0.05$) due to large standard deviations (figure 11).

The yield rate ratios of *C. vulgaris* based on chlorophyll-a concentration, dry weight, and biovolume concentration were higher than the yield rate ratios of *S. obliquus*. The ratios of the algae based on particle concentration were similar.

*Potassium*

There are no major differences between ratios of *S. obliquus* within the potassium experiment (table 4), and there were no significant differences between the yield rates based on chlorophyll-a concentration, particle concentration, and biovolume concentration of this algae on 500 mg $K^+$ $l^{-1}$, and its yield rates in WC medium ($p > 0.05$; figures 6, 12 & 13).

Also, the yield rate ratios based on chlorophyll-a concentration, particle concentration, and dry weight of *C. vulgaris* concerning the potassium experiment, were all similar and close to 1.00. Analysis showed that the yield rate based on particle concentration and particle volume of *C. vulgaris* at 500 mg $K^+$ $l^{-1}$ was not significanlty different from its yield rate and particle volume in WC medium (figures 12 & 16). However, the yield rate ratio based on biovolume concentration of *C. vulgaris* was only 0.34 for the potassium experiment, yet the yield rate of this alga at 500 mg $K^+$ $l^{-1}$ was comparable to the yield rate of *C. vulgaris* in WC medium ($p > 0.05$; figure 13). Yet, the mean yield rate of *C. vulgaris* in WC medium, which was used for calculation of the yield rate ratio, was higher than the mean yield rate at 500 mg $K^+$ $l^{-1}$. Still, the difference in yield rates was not significant due to the large standard deviation of the yield rate based on biovolume concentration in WC medium.

Except from the ratios based on biovolume, the yield rate ratios of *C. vulgaris* concerning the potassium experiment are slightly higher than the yield rate ratios of *S. obliquus* (table 4). Yet, the ratio based on biovolume of *S. obliquus* is three times as large as the ratio of *C. vulgaris*.

*Comparison between experiments*

Next to the difference in yield rate ratios of *C. vulgaris* within experiments, the ratios were also different between experiments. The yield rate ratios based on dry weight and particle concentration, showed that the ratios concerning the growth on the highest level of posphate (500 mg $PO_4^{3-}$ $l^{-1}$) and potassium (500 mg $K^+$ $l^{-1}$) were higher than the ratio concerning the ammonium experiment, for both *S. obliquus* and *C. vulgaris* (table 4). This was also true for the yield rate ratios based on biovolume concentration of *S. obliquus*. However, the yield rate ratio based on biovolume concentration of *C. vulgaris* concerning the potassium experiment was lower than the ratio of this alga concerning the ammonium experiment.

Also, all the yield rate ratios of *S. obliquus* concerning the potassium experiment were higher than the ratios of this alga for the phosphate experiment. On the other hand, except from the yield rate based on particle concentration, all ratios of *C. vulgaris* concerning the phosphate experiment were higher than, or similar to, the ratios for the potassium experiment.

For the manure experiment, only the ratios based on chlorophyll-a were calculated, and these were much lower than the yield rate ratios based on chlorophyll-a concentration of both algae for the experiments on 100 mg $NH_4^+$ $l^{-1}$, 500 mg $PO_4^{3-}$ $l^{-1}$, and 500 mg $K^+$ $l^{-1}$. However, the yield rate ratio of *C. vulgaris* was nearly three times as large as the ratio of *S. obliquus* for the manure experiment.

Growth of *C. vulgaris* and *S. obliquus* on high levels of nutrients compared to growth of these algal species on digested pig manure.

The digested pig manure that was used for the growth experiment of *S. obliquus* and *C. vulgaris* on digested pig manure, contained high levels of ammoniacal nitrogen, and dissolved phosphate; and was extremely rich in potassium (table 5).

**Table 5**. Properties of the used digested pig manure and groundwater. (n.a. = not available)

| | Digested manure | | Groundwater | |
|---|---|---|---|---|
| | Average | St. Dev. | Average. | St. Dev. |
| Electrical Conductivity (mS $cm^{-1}$) | 24,7 | n.a. | 0,167 | n.a |
| Total Nitrogen (N) (mg $l^{-1}$) | 1511 | 65,99 | n.a | n.a |
| Ammoniacal Nitrogen ($NH_3$-N) (mg $l^{-1}$) | 1246 | 8,94 | 0,17 | 0,01 |
| Total Phosphorus (P) (mg $l^{-1}$) | 46,8 | 1,9 | n.a | n.a |
| Dissolved Phosphorus ($PO_4$-P) (mg $l^{-1}$) | 41,86 | 1,03 | 0,10 | 0,00 |
| Potassium (K) (mg $l^{-1}$) | 28692 | 576 | 1,20 | 0,16 |
| Copper (Cu) (mg $l^{-1}$) | 0,37 | 0,05 | 0,00 | 0,00 |
| Zinc (Zn) (mg $l^{-1}$) | 0,28 | 0,04 | 0,01 | 0,00 |
| Iron (Fe) (mg $l^{-1}$) | 26,32 | 0,77 | 3,97 | 0,01 |

Dilution of the manure with groundwater lowered the nutrient levels to concentrations that enabled growth of *S. obliquus* and *C. vulgaris*. The concentrations of ammonium and phosphate in the medium containing 2.5% digested pig manure, which was chosen as the highest level of digested pig manure still allowing algal growth comparable to growth in WC medium, were lower than the concentrations of these nutrients in the media containing high levels of ammonium and phosphate (100 mg $NH_4^+$ $l^{-1}$, and 500 mg $PO_4^{3-}$ $l^{-1}$, respectively) (table 6). Furthermore, the salinity and electrical conductivity (EC) of the 2.5% digested pig manure dilution were also lower than the salinity and EC of the media containing 100 mg $NH_4^+$ $l^{-1}$, 500 mg $PO_4^{3-}$ $l^{-1}$, and 500 mg $K^+$ $l^{-1}$. And the salinity and EC of the dilution containing 5% digested pig manure was also lower than the salinity and EC of the media containing 500 mg $K^+$ $l^{-1}$. On the other hand, potassium levels in the medium containing 2.5% digested pig manure exceeded the potassium level in the medium having the highest level of potassium (500 mg $K^+$ $l^{-1}$).

**Table 6**. Properties of WC medium, the media containing the 'highest levels of nutrients', and the various dilutions of digested pig manure.

| Medium | EC (mS/cm) | Salinity | $NH_4^+$ (mg/l) | $PO_4^{3-}$ (mg/l) | $K^+$ (mg/l) |
|---|---|---|---|---|---|
| WC medium | 0.31 | 0 | 0.00 | 4.75 | 3.91 |
| 100 mg $NH_4^+$ $l^{-1}$ | 1.19 | 0.4 | 100 | 4.75 | 3.91 |
| 500 mg $PO_4^{3-}$ $l^{-1}$ | 1.36 | 0.5 | 0.00 | 500 | 3.91 |
| 500 mg $K^+$ $l^{-1}$ | 2.37 | 1 | 0.00 | 4.75 | 500 |
| 0% pig manure | 0,17 | 0 | 0,22 | 0,31 | 1,20 |
| 2.5% pig manure | 0,92 | 0.2 | 40,33 | 3,51 | 718,47 |
| 5% pig manure | 1,73 | 0.7 | 80,44 | 6,71 | 1435,74 |
| 25% pig manure | 7,50 | 4.1 | 401,33 | 32,32 | 7173,90 |
| 50% pig manure | 13,98 | 8.1 | 802,44 | 64,33 | 14346,60 |

## Discussion

Previous studies have shown that *S. obliquus* and *C. vulgaris* can be grown on diluted pig manure (Sevrin-Reyssac 1998; Travieso, Benítez et al. 2006), but it was not clear which of those two algal species was more suitable for commercial growth on pig manure. In order to learn more about the commercial applicability of these algae, *S. obliquus* and *C. vulgaris* were grown on high levels of ammonium, phosphate, potassium, and finally on various dilutions of digested pig manure.

<u>Determination of the yield rates of *Chlorella vulgaris* and *Scenedesmus obliquus*.</u>

Yield rates of *S. obliquus* and *C. vulgaris* were calculated using four different parameters: the chlorophyll-a concentration (µg chlorophyll-a $l^{-1}$), the particle concentration (counts $ml^{-1}$), the biovolume concentration ($\mu m^3$ biovolume $ml^{-1}$), and the dry weight (mg $l^{-1}$). In addition, the efficiency of photosystem II was measured, to examine the condition of the algae during the experiments.
The chlorophyll-a concentration can be influenced by photoacclimation (Falkowski, Dubinsky et al. 1985; Dubinsky and Stambler 2009), which means that algae adapt to high cell densities by increasing the amount of chlorophyll per cell. In turn, the particle concentration only shows the amount of particles per volume of culture, and not the amount of cells. If the particle concentration is measured and algae form colonies or algal cells are adhesive, larger particles are formed, and the number of cells will be underestimated. However, in combination with the dry weight and biovolume concentration measurements, the chlorophyll-a concentration and particle concentration give a detailed view of algal growth and development. For example, the chlorophyll-a concentration was divided by the biovolume concentration at day 9, yielded the micrograms of chlorophyll-a per cubic micrometer ($\mu m^3$). And although the standard deviations of the biovolume measurements were very large, it was perferred to used biovolume concentration over particle concentration, because the particle volume was not comparable within and between the experiments (figures 14, 15 & 16).

During the experiments, it turned out that *C. vulgaris* tended to stick to the walls of glass flasks. This might explain why the chlorophyll-a and particle concentration of *C. vulgaris* in WC medium did not increase on the first two to three days of the growth experiments on high levels of nutrients and various dilutions of digested pig manure. It could also explain why the chlorophyll-a concentrations of this alga were very low on the first couple of days of the experiments, while the start concentration of chlorophyll-a was 10 µg $l^{-1}$. All in all, the yield rates of *C. vulgaris* were underestimated by adhesion of the *Chlorella* cells to the walls of the Erlenmeyer flasks. Furthermore, *C. vulgaris* formed large particles in the manure dilutions containing 2.5% and 5% digested pig manure (figure 10). This might effect algal growth, but further research is needed to elucidate if this is indeed the case.
In addition, the volume of the medium decreased strongly in the course of the experiments, because during the experiment 5 to 6 10 ml samples were taken for measurements. Which means that the medium volume decreased from 100 ml at day 1, to 40 – 50 ml at day 9. This might have influenced algal physiology and growth.

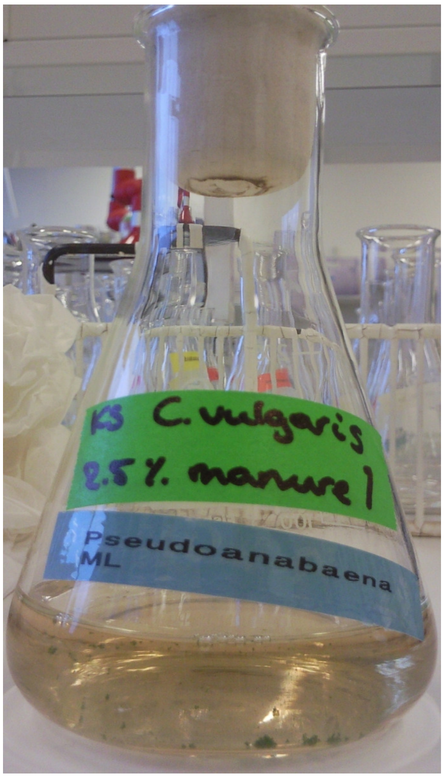


**Figure 10.** *C. vulgaris* formed large particles in the manure dilutions containing 2.5% digested pig manure. Flocs of *C. vulgaris* lie on the bottom of the Erlenmeyer flask.

The yield rates on WC medium for both *C. vulgaris* and *S. obliquus*, were not comparable between experiments. Since pre-culturing conditions and the growth conditions during all experiments were similar, it is not clear why the yield rates on WC medium were so different between experiments. However, although the algae were transferred to fresh WC medium four days before each experiment, the volume of culture that was transferred was not equal. Thus, it is possible that when the algae were transferred to the experimental media with high nutrient levels and manure, the physiological condition of the algae was different between experiments as a result from differences in cell density in the stock cultures. On the other hand, there were no differences in the efficiency of photosystem II, so the difference in yield rates on WC medium could also be caused by an unnoticed disturbance in the experimental conditions.
To be able to compare the results between experiments and between the different parameters of algal growth, the yield rate ratios were calculated.

<u>Yield rates based on chlorophyll-a concentration, particle concentration, and biovolume concentration of *C. vulgaris* and *S. obliquus* after growth on high nutrient levels.</u>

*Ammonium*
The ammonium levels that were used for the growth experiment of *S. obliquus* and *C. vulgaris* on high concentrations of ammonium (100 – 1000 mg $NH_4^+$ $l^{-1}$), were in the same range as those used by Tam and Wong (1996). However, Tam and Wong used concentrations of ammoniacal nitrogen (mg $NH_3$-N $l^{-1}$) while the concentrations used in this experiment were ammonium concentrations (mg $NH_4^+$ $l^{-1}$). Furthermore, the Tam and Wong replaced the nitrate component in the medium that was used as a basis for the media containing ammonium, by various concentrations of ammonium, whereas in this experiment, the various concentrations of ammonium were simply added to the WC medium.
The highest level of ammonium used by Tam and Wong (1000 mg N $l^{-1}$) was roughly half times the concentration of ammoniacal nitrogen in pig manure as described in literature (table 1). In theory, this would correspond to a dilution containing 50% pig manure, the highest level of pig manure that was used for the growth of *S. obliquus* and *C. vulgaris* in this experiment.

High levels of ammonium had a negative effect on growth of both *S. obliquus* and *C. vulgaris*. Even at the lowest concentration of ammonium that was used, 100 mg $NH_4^+$ $l^{-1}$, the yield rates based on chlorophyll-a concentration and particle concentration of both algae were lower than their yield rates in WC medium. This was also true for the yield rate based on biovolume concentration of *S. obliquus*, but the yield rate of *C. vulgaris* at 100 mg $NH_4^+$ $l^{-1}$ based on biovolume concentration was comparable to its yield rate in WC medium. Yet, the mean yield rates based on biovolume concentration of *C. vulgaris* at 100 mg $NH_4^+$ $l^{-1}$ and in WC medium were different, but due to large variances between replica's the difference was not significant.
However, Tam and Wong (1996) found that when *C. vulgaris* was grown at pH 7.0 and 20 °C for 21 days, there was no significant difference in growth at ammonium concentrations between 50 – 250 mg $NH_3$-N $l^{-1}$ when compared to the control treatment with nitrate. The dissimilarity in results between the experiment of Tam and Wong, and the current experiment, might have been caused by a dissimilarity in experimental conditions. For example, in the current experiment, WC medium (pH 7.5) was used as the control medium and as a basis for the experiment on high levels of ammonium, while Tam and Wong used a commercial Bristol medium (pH 7.0). Furthermore, the light levels used in the current experiment (11.57 µmol $s^{-1}$ $m^{-2}$) were rather low, and the amount of medium that was used per batch (start volume: 100 ml) was different from that of Tam and Wong (start volume: 150 ml). In addition, during the experiment of Tam and Wong, the algae were grown for 21 days in stead of 9 days, and they might have adapted themselves to high levels of ammonium (Przytocka-Jusiak, Duszota et al. 1984).

In the current experiment, the yield rate of *S. obliquus* based on chlorophyll-a concentration at 100 mg $NH_4^+$ $l^{-1}$ was significantly lower than the yield rate of *C. vulgaris*. However, at this concentration of ammonium, the yield rate based on particle concentration of *S. obliquus* was significanlty higher than the yield rate of *C. vulgaris*. No significant difference was found between the yield rates of *S. obliquus* and *C. vulgaris* based on biovolume, due to large variations in biovolume concentrations between replica's. Still, except from the yield rate ratios based on particle concentration, the yield rate ratios of *C. vulgaris* concerning the growth on high levels of ammonium were higher than the yield rate ratios of *S. obliquus* for that experiment.
In addition, the mean particle volume and mean amount of chlorophyll-a per $\mu m^3$ of *C. vulgaris* were higher than the mean particle volume and mean amount of chlorophyll-a per $\mu m^3$ of *S. obliquus*. The larger mean particle volume of *C. vulgaris* might explain why the yield rate based on particle concentration of *C. vulgaris* at 100 mg $NH_4^+$ $l^{-1}$ was lower than the yield rate of *S. obliquus*, since it suggests that less, but bigger particles of *C. vulgaris* were formed. Also, the higher mean amount of chlorophyll-a per $\mu m^3$ of *C. vulgaris* might account for the higher yield rate of this alga based on chlorophyll-a concentration at 100 mg $NH_4^+$ $l^{-1}$ compared to the yield rate of *S. obliquus*. Yet, it was not possible to substantiate foregoing explanations with statistical evidence, since the differences between the particle volumes and the amount of chlorophyll-a per $\mu m^3$ of *S. obliquus* and *C. vulgaris* were not significant, due to large standard deviations.

It is clear that high levels of ammonium are toxic to algae, and the concentration at which ammonium becomes toxic to algae varies with the algal species and experimental conditions. Azov and Goldmann (1982) found that the pH value of the culture medium had a great effect on the inhibition of $C^{14}$ uptake by $NH_4Cl$ addition for *S. obliquus*. At pH 8.0 the addition of 10 mM $NH_4Cl$ (approximately 180 mg $NH_4^+$ $l^{-1}$) led to a slight decrease in the uptake of $C^{14}$ compared to the control without $NH_4Cl$. However, 10 mM $NH_4Cl$ at pH 8.4 led to an $C^{14}$ uptake that was only 50% of the $C^{14}$ uptake in the control culture. Furthermore, when they calculated the $NH_3$ concentration from culture pH they found a curve relating to the ratio of $C^{14}$ uptake of the cultures compared to the $C^{14}$ uptake of the control culture. This curve showed that $C^{14}$ uptake was only 50% of the uptake in the control culture, when the culture medium contained 1.25 mM $NH_3$ (approximately 21 mg $NH_3$ $l^{-1}$). Thus, toxicity of ammonium is caused by toxicity of ammonium gas ($NH_3$), and dissociation of nontoxic $NH_4^+$ into $NH_3$ is pH dependent. Futhermore, Tam and Wong (1996) showed that the removal of ammonium by algae led to a decrease in pH values, which suggests that a high amount of $H^+$ ions were generated in media containing high levels of ammonium. A lower pH reduces the formation of ammonia gas, which means that most N exists in ammonium ion form. They state that because this form is less toxic to algae, it could explain why *C. vulgaris* could still grow at 1000 mg $NH_3$-N $l^{-1}$.
Prior to the growth experiment of *S. obliquus* and *C. vulgaris* on high levels of ammonium (0 – 1000 mg $NH_4^+$ $l^{-1}$), the pH of the culture media was set at 7.5. However, algal growth influences the pH of the culture medium by uptake of $CO_2$ or $HCO_3^-$ (Myers 1951), and since no control of the pH took place during the growth period of 9 days, it is unknown what the pH of the culturing media were after 9 days of algal growth. Therefore, it is unknown what part of the $NH_4^+$ that was present was dissociated into toxic $NH_3$.

*Phosphate*
The highest level of phosphate that was used for this experiment (500 mg $PO_4^{3-}$ $l^{-1}$) was based on roughly half times the concentration of total phosphorus in pig manure as described in literature (table 1). In theory, this would correspond to a dilution containing 50% pig manure, the highest level of pig manure that was used for the growth of *S. obliquus* and *C. vulgaris* in this experiment. The other levels of phosphate were chosen to cover the broad range between 0 mg $PO_4^{3-}$ $l^{-1}$ and 500 mg $PO_4^{3-}$ $l^{-1}$.

After sterilization, a white precipitation was visible in the media containing high levels of phosphate, 25 – 500 mg $PO_4^{3-}$ $l^{-1}$. This probably resulted from precipitation of phosphate with calcium, iron, and magnesium in the media. And because of this, it is not clear what the true phosphate levels were in the media.
The absence of phosphate in the medium containing 0 mg $PO_4^{3-}$ $l^{-1}$ limited algal growth. Yet, algal growth was not zero, since some phosphate was transferred when the medium was inoculated with the *C .vulgaris* and *S. obliquus* from the algae stocks. Furthermore, the materials that were used, were not phosphate free. The small amount of phosphate that was available led to algal growth. But the photosystem II efficiency of both algal species declined during the experiment at 0 mg $PO_4^{3-}$ $l^{-1}$, indicating that *S. obliquus* and *C. vulgaris* experienced physiological stress in the absence of phosphate.

At 500 mg $PO_4^{3-}$ $l^{-1}$ the yield rate of *C. vulgaris* based on chlorophyll-a concentration was significantly higher than the yield rate of *S. obliquus*. In contrast, the yield rate of *C. vulgaris* based on particle concentration at 500 mg $PO_4^{3-}$ $l^{-1}$ was significanlty lower than the yield rate of *S. obliquus*. However, the mean yield rate based on biovolume concentration of *C. vulgaris* at 500 mg $PO_4^{3-}$ $l^{-1}$ was higher than the mean yield rate based on biovolume of *S. obliquus*, but the difference was not significant, due to large variances in measured biovolume concentration between replica's. Furthermore, the yield rate ratios of *C. vulgaris* were, except from the ratio based on particle concentration, higher than the ratios of *S. obliquus*.
At 500 mg $PO_4^{3-}$ $l^{-1}$, the particle volume of *C. vulgaris* was significantly larger than its particle volume in WC medium, while the amount of chlorohpyll-a per $\mu m^3$ of *C. vulgaris* at 500 mg $PO_4^{3-}$ $l^{-1}$ was comparable to its amount of chlorophyll-a per $\mu m^3$ in WC medium. Thus, the fact that growth of *C. vulgaris* on 500 mg $PO_4^{3-}$ $l^{-1}$ led to less, but bigger particles could explain why the yield rate of *C. vulgaris* based on particle concentration declined at high phosphate levels, while its yield rate based on chlorophyll-a concentration increased.
On the other hand, the particle volume and amount of chlorophyll-a per $\mu m^3$ of *S. obliquus* at 500 mg $PO_4^{3-}$ $l^{-1}$ were comparable to its particle volume and amount of chlorophyll-a per $\mu m^3$ in WC medium. Thus, the decline in yield rates based on particle concentration of *S. obliquus* was not caused by the formation of larger particles. Furthermore, the yield rates of *S. obliquus* based on chlorophyll-a and also decreased at high phosphate levels, as did its mean yield rates based on biovolume concentrations. Yet, the decrease of the latter was not significant, due to large differences in biovolume concentration between replica's. Growth of *S. obliquus* at 500 mg $PO_4^{3-}$ $l^{-1}$ might have been limited by depletion of trace elements in the growth medium. As described above, a white precipitation formed after sterilization. Precipitation of iron, calcium and magnesium with phosphate would render these trace elements, that are important for algal physiology and growth, unusable for the algae, and could therefore impede algal growth. Furthermore, Walach (1986) reported an increase of adhesion of *C. vulgaris* to glass surfaces when the concentrations of iron, calcium, and trace elements were lowered. The increase in particle size of *C. vulgaris* at high levels of phosphate might be caused by algal cells sticking together, as a response to decreased levels of readily available trace elements. Further research is needed to validate this theory.

*Potassium*
Like the highest level of phosphate, the highest level of potassium that was used for this experiment (500 mg $K^+$ $l^{-1}$) was based on roughly half times the concentration of total phosphorus in pig manure as described in literature (table 1). The other levels of potassium were chosen to cover the broad range between 0 mg $K^+$ $l^{-1}$ and 500 mg $K^+$ $l^{-1}$.
In the absence of potassium, the yield rates based on chlorophyll-a concentration of both *C. vulgaris* and *S. obliquus* were lower than the yield rates of both algae in WC medium. Also the decrease in photosystem II efficiency of *C. vulgaris* and *S. obliquus* during the experiment, suggests that the absence of potassium was limiting photosynthesis. This might be explained by the fact that potassium is needed for photosynthesis, and it is an activator of a large number of enzymes

(Shukla and Chand Rai 2006). Yet, the yield rate based on particle concentration of *C. vulgaris* at 0 mg $K^+$ $l^{-1}$ was comparable to its yield rate in WC medium. However, the particle volume of *C. vulgaris* in the absence of potassium was significanlty smaller than its particle volume in WC medium. Still, the mean yield rates based on biovolume concentration of both *S. obliquus* and *C. vulgaris* at 0 mg $K^+$ $l^{-1}$ were not significantly different from their yield rates in WC medium, due to large variances in measurements of the replica's.
Between high levels of potassium, 25 – 500 mg $K^+$ $l^{-1}$, the yield rates based on chlorophyll-a concentration and particle concentration of both algal species were higher than or comparable to their yield rates in WC medium. Furthermore, the yield rate ratios based on chlorophyll-a, dry weight, and particle concentration of *S. obliquus* and *C. vulgaris* were 1.00 or close to 1.00. However, the yield rate ratio of *C. vulgaris* based on biovolume concentration was only 0.34. Yet, aside from from the latter, the results suggest that potassium levels of 25 – 500 mg $K^+$ $l^{-1}$ are not toxic to *S. obliquus* and *C. vulgaris*.
The yield rates based on chlorophyll-a concentration, particle concentration, and biovolume concentration of *S. obliquus* were significantly higher than *Chlorella*'s yield rates at 500 mg $K^+$ $l^{-1}$, which suggests that *S. obliquus* is more suitable for growth on high potassium levels than *C. vulgaris*. However, the yield rates of *C. vulgaris* based on chlorophyll-a concentration and particle concentration in WC medium were also much lower, and the yield rate ratios of *C. vulgaris* based chlorophyll-a concentration, dry weight, and particle concentration were actually higher than the ratios of *S. obliquus*. In addition, the yield rate ratio of *C. vulgaris* based on dry weight, was also higher than the yield rate ratio of *S. obliquus*.
Thus, it depends on how the yield rates concerning the potassium experiment are compared. If the absolute yield is compared, than the yield of *S. obliquus* at 500 mg $K^+$ $l^{-1}$ is significanlty higher than the yield of *C. vulgaris* within a certain period of time, indicating that *S. obliquus* is more suitable for growth on high potassium levels. On the other hand, comparison of the yield rates of both algae at 500 mg $K^+$ $l^{-1}$ to their yield rates in WC medium suggests that *C. vulgaris* is more suitable for growth on high potassium levels.

*Comparison between experiments*
The fact that the yield rate ratios of *S. obliquus* and *C. vulgaris* concerning the ammonium were lower than the yield rate ratios of these algae for the growth experiments on phosphate and potassium, indicates that high levels of ammonium (100 mg $NH_4^+$ $l^{-1}$) are more inhibiting to *S. obliquus* and *C. vulgaris*, than 500 mg $PO_4^{3-}$ $l^{-1}$ and 500 mg $K^+$ $l^{-1}$. Furthermore, the yield rate ratios of *S. obliquus* on high levels of phosphate were lower than the ratios on high levels of potassium, indicating that the high levels of phosphate are more restraining to *S. obliquus* than high levels of potassium. Yet, the latter might not be directly caused by high phosphate levels, but could be a result from depletion of readily available trace elements within the medium due to their precipitation with phosphate.

<u>Yield rates based on chlorohpyll-a concentration of *C. vulgaris* and *S. obliquus* after growth on various dilutions of digested pig manure.</u>

The range of digested pig manure dilutions that was used for the growth experiment of *S. obliquus* and *C. vulgaris* on high levels of digested pig manure (up to 50% digested pig manure), was based on the range of pig slurry dilutions that was used by Gantar *et al*. (1991). The dilutions of digested pig manure, 0%, 2.5%, 5%, 25%, and 50%, were chosen to cover both the lower, the mean, and the higher sections of this range.
Both the yield rates and the yield rate ratios of *C. vulgaris* and *S. obliquus* on 2.5% digested pig manure, were lower than their yield rates and yield rate ratios on high levels of ammonium, phosphate, and potassium. Furthermore, at dilutions containing 25% digested pig manure, no increase in chlorophyll-a was measured, and no algal cells were found during microscopic analysis of the samples. Yet, Gantar *et al*. (Gantar, Obreht et al. 1991) report that in the first 12 days of their experiment *S. quadricauda* could grow and even compete with algal species within the manure at

manure dilutions up to 30%. However, both the used algal species as the experimental conditions and setup were different from the current experiment. For example, Gantar grew *S. quadricauda* under day light, at a temperature of 25 °C, Also, they used the liquid phase of pig manure, while digested pig manure was used for the experiment descibed in this report. An imporant difference between these two forms of pig manure is the amount of dissolved phosphorus (P-$PO_4^{3-}$) within the manure. The level of dissolved phosphorus described by Gantar *et al*. in the liquid phase of pig manure is 17.0 mg $l^{-1}$, whereas the level of dissolved phosphorus in the digested pig manure was 41.9 mg $l^{-1}$. Still, the higher level of dissolved phosphorus in the dilution containing 25% digested pig manure do not correspond to a phosphate level that fully restrained algal growth (tabel 6).
In addition, the electrical conductivity and salinity of the dilution containing 2.5% digested pig manure were lower than these values in the media containing 100 mg $NH_4^+$ $l^{-1}$, 500 mg $PO_4^{3-}$ $l^{-1}$, and 500 mg $K^+$ $l^{-1}$. Also, the levels of ammonium and phosphate were much lower in 2.5% digested pig manure, compared to the media containing the 'highest levels' of ammonium and phosphate. Thus, it is unlikely that these nutrients were toxic to the algae, or that the concentration of salts was to high for algal growth. However, the level of potassium in 2.5% digested pig manure, 718.47 mg $K^+$ $l^{-1}$, exceeded the concentrations that were used in the growth experiment on high levels of potassium (25 – 500 mg $K^+$ $l^{-1}$). It could be possible that potassium becomes toxic to *C. vulgaris* and *S. obliquus* at these levels.
Research by Shu-Ying *et al.* (1999) showed that high levels of $K^+$ (higher than 50 mM, or 1955 mg $K^+$ $l^{-1}$) led to ultrastructural changes in the chloroplasts and inhibition growth of *Dunaliella salina*, and these changes had an inhibitory effect on photosynthesis and the overall growth of this alga. *D. salina* lives in marine environments and is very halotolerant, thus potassium might become inhibiting to freshwater algal species, like *C. vulgaris* and *S. obliquus*. Future research will have to elucidate wether high levels of potassium (above 500 mg $K^+$ $l^{-1}$) inhibit photosynthesis and growth of algae *S. obliquus* and *C. vulgaris*.

Still, there are a lot of other factors in manure that could play a role in decreasing the yield rates of both *S. obliquus* and *C. vulgaris*. For instance, the digested pig manure contained the heavy metals copper and zinc. These might be toxic to algae. The dilution containing 2.5% digested pig manure contained 9.25 µg Cu $l^{-1}$, and 7.00 µg Zn $l^{-1}$. These concentrations are much lower than concentrations of copper and zinc that are known to be toxic to *C. vulgaris* and *S. obliquus* (Lam, Wut et al. 1999; Yan and Pan 2002; Awasthi and Das 2005; Monteiro, Fonseca et al. 2011). But toxicity of heavy metals is pH dependent, and therefore the concentrations at which copper and zinc become toxic differ with the experimental conditions. Furthermore, other toxic heavy metals might also be present in the manure, like lead (Pb) and cadmium (Cd) (Nicholson, Chambers et al. 1999; Moral, Perez-Murcia et al. 2008), but these were not measured. Combination of these heavy metals with copper and zinc might influence the concentration at which these compounds become toxic to algae (Lam, Wut et al. 1999). Also, 100% digested pig manure contained 26.32 mg iron per liter, and the dilution of 2.5% digested pig manure in groundwater contained 4.53 mg iron per liter. Iron is necessary for photosynthesis, but high levels of iron could lead to stress and inhibition of algal growth (Estevez, Malanga et al. 2001). However, Estevez *et al.* (2001) have shown that addition of iron up to 90 µM to the incubation medium increased biomass of *C. vulgaris*. Therefore, it is likely that the 4.53 mg iron per liter (81 µM) which is present in the dilution of 2.5% digested pig manure in groundwater, is not toxic to *C. vulgaris*.
Another important factor in dilutions of digested pig manure is the color of the manure. Groundwater was very clear and nearly colorless, whereas 100% manure was very dark brown. The dilution containing 2.5% digested pig manure was yellow-brownish, and it is possible that light in the red and blue part of the spectrum is absorbed by this coloration. De Pauw et al. (1980) described that the algal biomass yields obtained in cultures with manure were on average about 20 to 30% lower as those of the cultures with inorganic fertilizers. They attributed this decrease in yields to the dark color of the manure. In addition, Saeys *et al*. (2005) described the potential of visible and near-infrared reflectance spectroscopy as a method to analyse pig manure onsite and online. They

found that in the visible region, there was a high overall absorption which was higher in the blue region (around 475 nm) than in the red region (around 650 nm), which resulted from the dark brown colour of the manure. This indicates that manure coloration has an effect on light penetration, and even at relatively low concentrations of manure (2.5% digested pig manure) it might have a significant effect on photosynthesis.

Implications for commercial growth of *S. obliquus* or *C. vulgaris* on digested pig manure.

The results indicate that both *C. vulgaris* and *S. obliquus* can grow on high levels of nutrients, but the yield rate and yield rate ratio of *C. vulgaris* were higher than the yield rate and yield rate ratio of *S. obliquus* when the algae were grown on 2.5% digested pig manure. Furthermore, in general the yield rate ratios of *C. vulgaris* concerning the ammonium experiment was higher than the yield rate ratios of *S. obliquus*, indicating that ammonium is more toxic to *S. obliquus* than to *C. vulgaris*. This characteristic of *C. vulgaris* is advantegous when algae are grown on pig manure, since manure in general contains a lot of nutrients, and is particularly rich in ammonium (Voermans and Asseldonk 1992; Aarnink and Verstegen 2007).
In addition, when the cultures of *C. vulgaris* were not stirred, this alga tended to stick to the walls of the flasks. And the increase in particle size of *C. vulgaris* during the growth experiments on phosphate, and formation of large particles of *C. vulgaris* in digested pig manure, suggests that *C. vulgaris* cells also stick together in certain conditions. This might be an advantage for commercial algae growth, since larger particles make the harvesting process easier, because the algae could be harvested through filtration. However, the fact that *C. vulgaris* is also adhesive to glass and plastic when cultures are not stirred constantly, might also cause clogging of the bioreactor. For commercial growth it would mean that the bioreactor needs to be cleaned up after a certain amount of time, and thus, the process of growing algae would have to be interrupted.
Coloration of the pig manure might absorb red and blue light, even when relatively low concentrations of manure are used. This light is important for photosynthesis, and thus for algal growth. One way to improve the transmission of light through the dilution, is to dilute the pig manure even further. The results showed that *C. vulgaris* can grown on groundwater, but that growth is stimulated by adding 2.5% pig manure. These are promising results for the commercial growth of algae on diluted pig manure.

Concluding remarks and recommendations.

The level of 100 mg $NH_4^+$ $l^{-1}$ negatively effected the yield rates of both *C. vulgaris* and *S. obliquus*, while 500 mg $PO_4^{3-}$ $l^{-1}$ and 500 mg $K^+$ $l^{-1}$ were less inhibiting to algal growth. The presented results do not point out unanimously which algae is more suitable for growth on high levels of nutrients. Yet, both the yield rate and the yield rate ratio based on chlorophyll-a concentration of *C. vulgaris* was significantly higher than the yield rate and yield rate ratio of *S. obliquus* when grown on 2.5% digested pig manure. In addition, the fact that *C. vulgaris* tends to form larger particles when grown on manure is advantageous for the commercial applicability of this alga, since it makes the harvesting process easier. However, the adhesion of *C. vulgaris* might also cause clogging of the culture system.

This research shed light on the potential of the use of *S. obliquus* and *C. vulgaris* for commercial algal growth on pig manure, but it also showed were there is still room for improvement concerning the research on this subject. If these experiments were to be repeated, some adjustments to the methods should be made. For instance, to get more insight into the growth of *C. vulgaris* and *S. obliquus* on high levels of phosphate, it is recommendable to first sterilize the culture media without phosphate and the phosphate stock solution, and add the phosphate to the culture media after sterilization. This might prevent the precipitation of phosphate with other ions in the media. Furthermore, in the current experiment, the batch cultures where too small to produces replicas for

the dry weight measurements. To gain more insight into the biomass yield of algae on high levels of nutrients and dilutions of digested pig manure, the batch culture size should be large enough to produce replicas for dry weight measurements. In addition, pH was not controlled or measured during the growth period of 9 days. Monitoring the pH in future research could give more insight in the growth of the algae and on possible toxicity of nutrients (like ammonia gas). Measurements of nutrient uptake could also increase the knowledge on algal growth on various levels of ammonium, phosphate, and potassium.
Future research on growth of *C. vulgaris* and *S. obliquus* should include the growth of these algae on dilutions containing digested pig manure up to 5% instead of 50%. The optimum for growth of *C. vulgaris* and *S. obliquus* on digested pig manure lies in these relatively low concentrations of pig manure. In addition, it would be more logical to set up future experiments as bioassays, which would mean: more replicas that should be incubated at higher temperature and light for a shorter period of time (72 hours) in an incubator under continuous shaking. In addition, if the manure is not sterilised, the particle concentration and biovolume concentration should be analysed by microscopic analysis, to make sure the results are not biased by growth of bacteria.
Furthermore, the digested pig manure was filtered by means of ultrafiltration, which also eliminated a great part of the bacteria from the manure. Yet, the used groundwater was filtered with Whatman GF/C filters. These filters have a particle retention of 1.2 µm. In the future, if the groundwater is not sterilised, it should be filtered with filters having a smaller pore size. In this way, bacteria that could compete with the algae for nutrients and might pose a threat to animal welfare, will be filtered out.
All in all, there is still much to explore concerning the growth of *C. vulgaris* and *S. obliquus* on dilutions of digested pig manure in groundwater, but the perspectives of commercial growth of these algae on pig manure are promising.

## References


*Literature*

Aarnink, A. J. A. and M. W. A. Verstegen (2007). "Nutrition, key factor to reduce environmental load from pig production." Livestock Science **109**: 194-203.

Awasthi, M. and D. N. Das (2005). "Heavy metal stress on growth, photosynthesis and enzymatic activities of free and immobilized *Chlorella vulgaris*." Annals of Microbiology **55**(1): 1-7.

Azov, Y. and J. C. Goldman (1982). "Free Ammonia Inhibition of Algal Photosynthesis in Intensive Cultures." Applied and Environmental Microbiology **43**(4): 735-739.

Becker, E. W. (1984). "Biotechnology and Exploitation of the Green Alga *Scenedesmus obliquus* in India." Biomass **4**: 1-19.

Becker, E. W. (1994). Microalgae: Biotechnology and Microalgal biotechnology. History and uses of algae, Cambridge University Press**:** 293.

Béline, F., J. Martinez, et al. (1998). "Nitrogen transformations during anaerobically stored $^{15}$N-labelled pig slurry." Bioresource Technology **64**: 83-88.

Borowitzka, M. A. (1999). "Commercial production of microalgae: ponds, tanks, tubes and fermenters." Journal of Biotechnology **70**: 313-321.

Converti, A., A. A. Casazza, et al. (2009). "Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production." Chemical Engineering and Processing **48**: 1146-1151.

De La Noue, J. and N. De Pauw (1988). "The potential of microalgal biotechnology: A review of production and uses of Microalgae." Biotech. Adv. **6**: 725-770.

Dubinsky, Z. and N. Stambler (2009). "Photoacclimation processes in phytoplankton: mechanisms, consequences, and applications." Aquatic Microbial Ecology **56**(2-3): 163-176.

Estevez, M. S., G. Malanga, et al. (2001). "Iron-dependent oxidative stress in *Chlorella vulgaris*. ." Plant Science **161**: 9-17.

Falkowski, P. G., Z. Dubinsky, et al. (1985). "Growth-Irradiance Relationships in Phytoplankton." Limnology and Oceanography **30**(2): 311-321.

Gantar, M., Z. Obreht, et al. (1991). "Nutrient Removal and Algal Succession during Growth of *Spirulina platensis* and *Scenedesmus quadricauda* on Swine Wastewater." Bioresource Technology **36**: 167-171.

Gantar, M. and Z. Svirčev (2008). "Microalgae and cyanobacteria: Food for thought." Journal of Phycology **44**: 260-268.

Grant, W. D., W. E. Mwatha, et al. (1990). "Alkaliphiles: ecology, diversity and applications." FEMS Microbiol. Rev. **75**: 255-270.

Greque de Morais, M. and J. A. Vieira Costa (2007). "Carbon dioxide fixation by *Chlorella kessleri*, *C. vulgaris*, *Scenedesmus obliquus* and *Spirulina* sp. cultivated in flasks and vertical tubular photobioreactors." Biotechnol. Lett. **29**: 1349-1352.

Hodaifa, G., M. E. Martínez, et al. (2010). "Influence of temperature on growth of *Scenedesmus obliquus* in diluted olive mill wastewater as culture medium." Eng. Life Sci. **10**(3): 257-264.

Iwasa, M., M. Yamamoto, et al. (2002). "Spirulina-Associated Hepatotoxicity." AJG: 3212-3213.

Jongbloed, A. W. and N. P. Lenis (1993). Excretion of nitrogen and some minerals by livestock. First International Symposium on Nitrogen Flow in Pig Production and Environmental Consequences, Wageningen, Netherlands.

Kerr, B. J., C. J. Ziemer, et al. (2006). "Manure composition of swine as affected by dietary protein and cellulose concentrations." Journal of Animal Science **84**: 1584-1592.

Kim, M. K., J. W. Park, et al. (2007). "Enhanced production of *Scenedesmus* spp. (green microalgae) using a new medium containing fermented swine wastewater." Bioresource Technology **98**: 2220-2228.

Lam, P. K. S., P. F. Wut, et al. (1999). "Individual and Combined Effects of Cadmium and Copper on the Growth Response of *Chlorella vulgaris*. ." Environmental Toxicology **14**(3): 347-353.

Lee, Y.-K. (2001). "Microalgal mass culture systems and methods: Their limitation and potential." Journal of Applied Phycology **13**: 307-315.

Liang, Y., N. Sarkany, et al. (2009). "Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions." Biotechnol. Lett. **31**: 1043-1049.

Litchfield, J. H. (1980). "Microbial Protein Production." Bioscience **30**(6): 387-396.

Lurling, M. and W. Beekman (2006). "Palmelloids formation in *Chlamydomonas reinhardtii*: defence against rotifer predators?" Ann. Limnol. - Int. J. Lim. **42**(2): 65-72.

Martínez, M. E., S. Sánchez, et al. (2000). "Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*." Bioresource Technology **73**: 263-272.

Maxwell, D. P., S. Falk, et al. (1994). "Growth at Low Temperature Mimics High-Light Acclimation in Chlorella vulgaris." Plant Physiol. **105**: 535-543.

Mazokopakis, E. E., C. M. Karefilakis, et al. (2008). "Acute rhabdomyolysis caused by Spirulina (*Arthrospira platensis*)." Phytomedicine **15**: 525-527.

Monteiro, C. M., S. C. Fonseca, et al. (2011). "Toxicity of cadmium and zinc on two microalgae, *Scenedesmus obliquus* and *Desmodesmus pleiomorphus*, from Northern Portugal." Journal of Applied Phycology **23**: 97-103.

Moral, R., M. D. Perez-Murcia, et al. (2005). "Estimation of nutrient values of pig slurries in Southeast Spain using easily determined properties." Waste Management **25**: 719-725.

Moral, R., M. D. Perez-Murcia, et al. (2008). "Salinity, organic content, micronutrients and heavy metals in pig slurries from South-eastern Spain." Waste Management **28**: 367-371.

Myers, J. (1951). "Physiology of the Algae." Annu. Rev. Microbiol. **5**: 157-180.

Nicholson, F. A., B. J. Chambers, et al. (1999). "Heavy metal contents of livestock feeds and animal manures in England and Wales." Bioresource Technology **70**: 23-31.

Pauw, N. d., H. Verlet, et al. (1980). Heated and unheated outdoor cultures of marine algae with animal manure. Algae biomass: production and use. G. Shelef and C. J. Soeder. Amsterdam, Elsevier North Holland**:** 315-341.

Przytocka-Jusiak, M., M. Duszota, et al. (1984). "Intensive culture of *Chlorella vularis*/AA as the second stage of biological purification of nitrogen industry wastewaters." Water Res. **18**: 1-7.

Rellán, S., J. Osswald, et al. (2009). "First detection of anatoxin-a in human and animal dietary supplements containing cyanobacteria." Food and Chemical Toxicology **47**: 2189-2195.

Saeys, W., A. M. Mouazen, et al. (2005). "Potential for Onsite and Online Analysis of Pig Manure using Visible and Near Infrared Reflectance Spectroscopy." Biosystems Engineering **91**(4): 393-402.

Saeys, W. and H. Ramon Rapid analysis of hog manure using visual and near-infrared reflectance spectroscopy. Heverlee.

Sevrin-Reyssac, J. (1998). "Biotreatment of swine manure by production of aquatic valuable biomasses." Agriculture, Ecosystems and Environment **68**: 177-186.

Shu-Ying, M., H. Yang-Cheng, et al. (1999). "Effects of High $K^+$ and Alkaline pH on Ultrastructure of *Dunaliella salina* Chlorophlasts." Acta Botanica Sinica **41**(12): 1342-1344.

Shukla, B. and L. Chand Rai (2006). "Potassium-induced inhibition of photosynthesis and associated electron transport chain of *Microcystis*: Implication for controlling cyanobacterial blooms." Harmful Algae **5**: 184-191.

Soeder, C. J. and E. Hegewald (1992). *Scenedesmus*. Microalgal Biotechnology. M. A. Borowitzka and L. J. Borowitzka. Cambridge, Cambridge University Press**:** 59-88.

Soong, P. (1980). "Production and development of *Chlorella* and *Spirulina* in Taiwan." Algae Biomass: 97-113.

Suresh, A., H. L. Choi, et al. (2009). "Swine Slurry Characterization and Prediction Equations for Nutrients on South Korean Farms." Transactions of the ASABE **52**(1): 267-273.

Tam, N. F. Y. and Y. S. Wong (1996). "Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media." Bioresource Technology **57**: 45-50.

Travieso, L., F. Benítez, et al. (2006). "Batch mixed culture of Chlorella vulgaris using settled and diluted piggery waste." Ecological Engineering **28**: 158-165.

Vijayavel, K., C. Anbuselvam, et al. (2007). "Antioxidant effect of the marine algae *Chlorella vulgaris* against naphthalene-induced oxidative stress in albino rats." Mol. Cell. Biochemistry **303**: 39-44.

Voermans, J. A. M. and M. M. L. Asseldonk (1992). De koude vergisting van varkensmest. *Psychrophilic digestion of pig slurry*. Rosmalen, Proefstation voor de Varkenshouderij**:** 1-32.

Vonshak, A. (1990). "Recent advances in microalgal biotechnology." Biotech. Adv. **8**: 709-727.

Vonshak, A. and A. Richmond (1988). "Mass Production of the Blue-green Alga *Spirulina*: An Overview." Biomass **15**: 233-247.

Walach, M. R. (1986). "The growth and adhesion to glass of algal (*Chlorella*) cells: the effects of iron and other mineral nutrients." Appl. Microbiol. Biotechn. **24**: 468-470.

Walker, T. L., S. Purton, et al. (2005). "Microalgae as bioreactors." Plant Cell Rep. **24**: 629-641.

Wood, A. M., R. C. Everroad, et al. (2005). When to measure growth rates. Algal Culturing Techniques. R. A. Andersen. San Diego, Elsevier Academic Press**:** 281.

Yan, H. and G. Pan (2002). "Toxicity and bioaccumulation of copper in three green microalgal species." Chemosphere **49**: 471-476.

*Websites*
http://www.algae.wur.nl/
http://www.eurolab.nl/meststof-organisch-v.htm
http://www.lgpm.ecp.fr
http://www.microscopy-uk.org.uk

## Appendix 1 - [Chlorophyll-a] vs. Time

Growth experiment on various levels of ammonium.

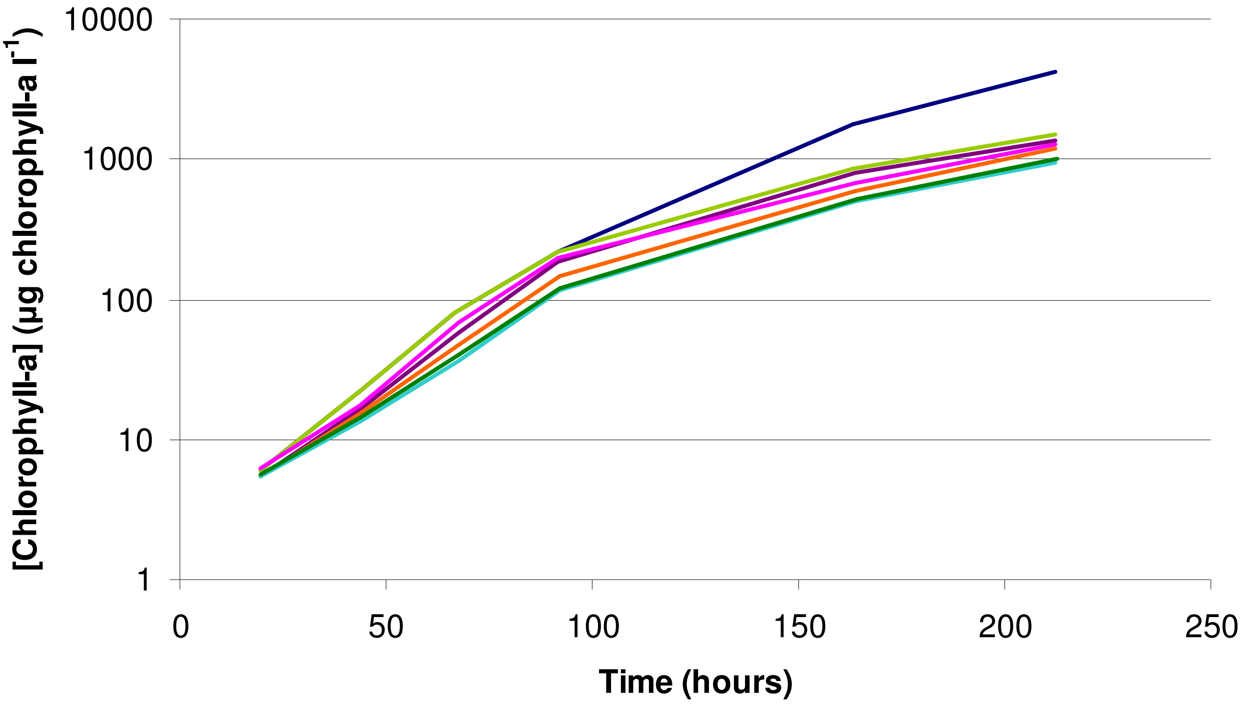


**Figure 1.1.** The mean chlorophyll-a concentration of *S. obliquus* plotted against time at different levels of ammonium: WC medium (dark blue), 100 mg $NH_4^+$ $l^{-1}$ (light green), 200 mg $NH_4^+$ $l^{-1}$ (purple), 400 mg $NH_4^+$ $l^{-1}$ (pink), 600 mg $NH_4^+$ $l^{-1}$ (orange), 800 mg $NH_4^+$ $l^{-1}$ (light blue), and 1000 mg $NH_4^+$ $l^{-1}$ (dark green).

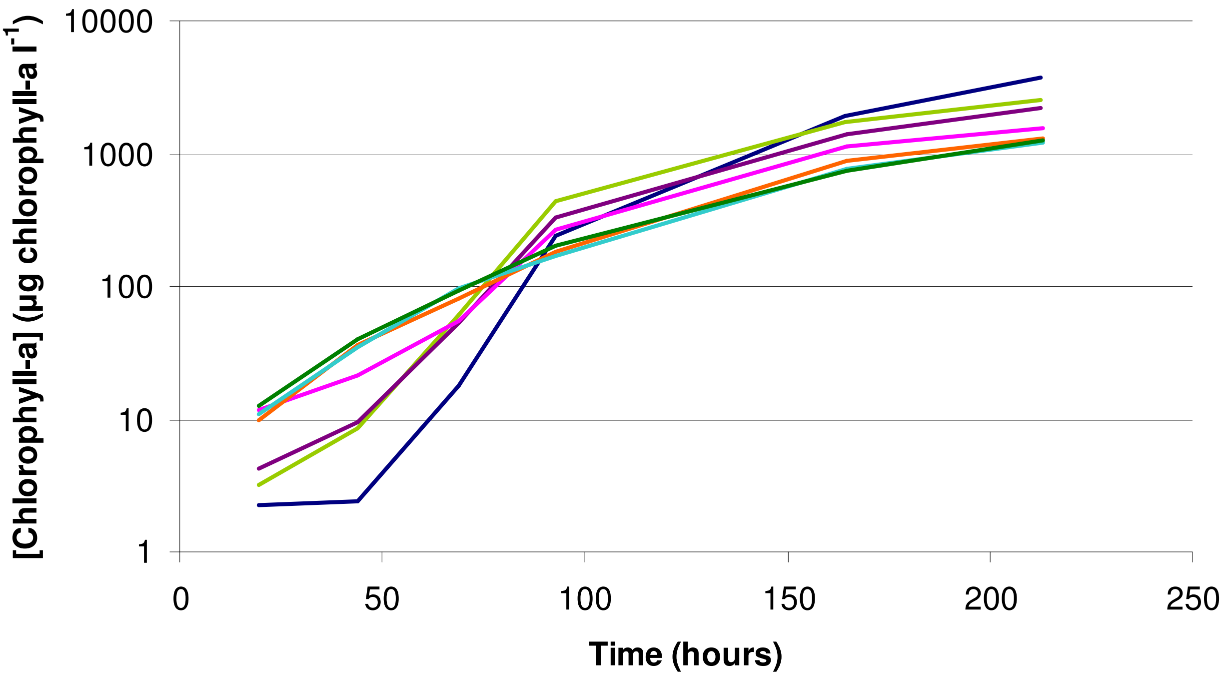


**Figure 1.2.** The mean chlorophyll-a concentration of *C. vulgaris* plotted against time at different levels of ammonium: WC medium (dark blue), 100 mg $NH_4^+$ $l^{-1}$ (light green), 200 mg $NH_4^+$ $l^{-1}$ (purple), 400 mg $NH_4^+$ $l^{-1}$ (pink), 600 mg $NH_4^+$ $l^{-1}$ (orange), 800 mg $NH_4^+$ $l^{-1}$ (light blue), and 1000 mg $NH_4^+$ $l^{-1}$ (dark green).

Growth experiment on various levels of phosphate.

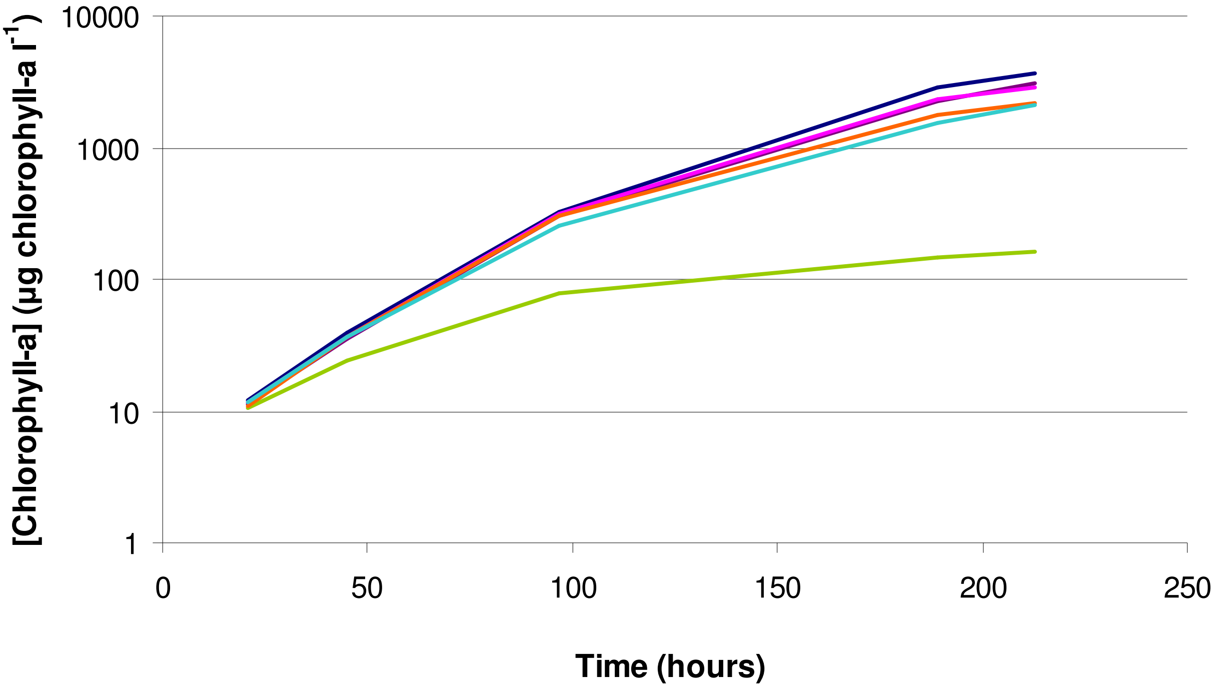


**Figure 2.1.** The mean chlorophyll-a concentration of *S. obliquus* plotted against time at different levels of phosphate: WC medium (dark blue), 0 mg $PO_4^{3-}$ $l^{-1}$ (light green), 25 mg $PO_4^{3-}$ $l^{-1}$ (purple), 50 mg $PO_4^{3-}$ $l^{-1}$ (pink), 200 mg $PO_4^{3-}$ $l^{-1}$ (orange), and 500 mg $PO_4^{3-}$ $l^{-1}$ (light blue).

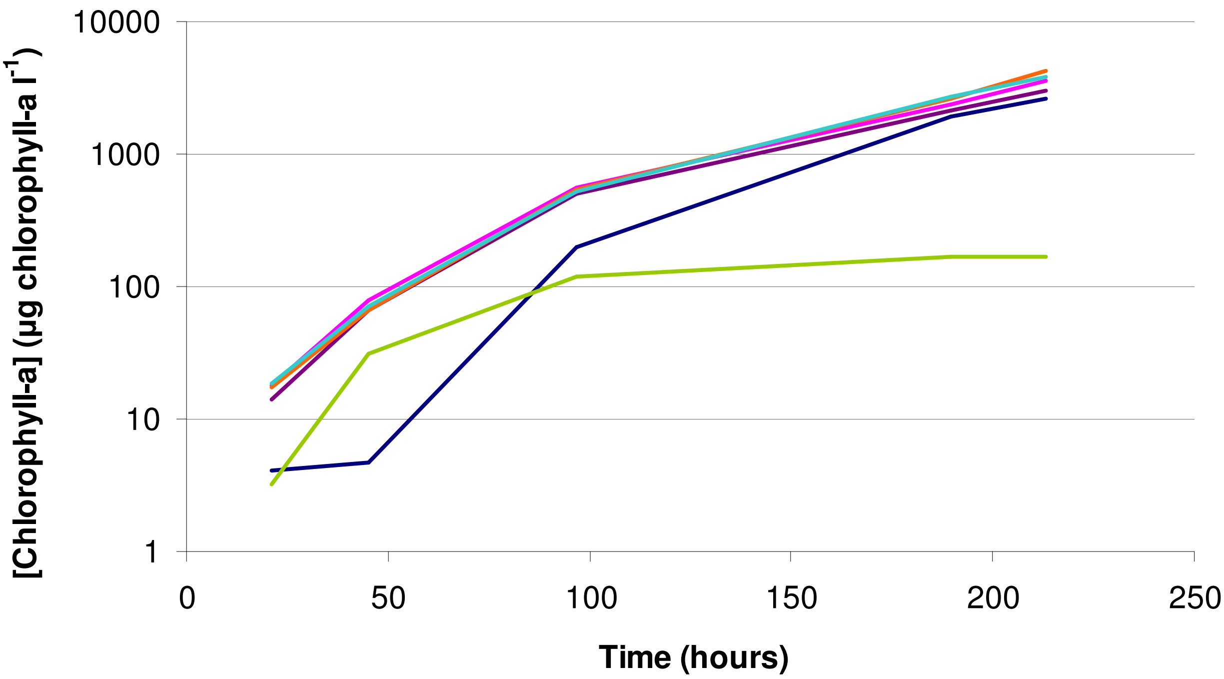


**Figure 2.2.** The mean chlorophyll-a concentration of *C. vulgaris* plotted against time at different levels of phosphate: WC medium (dark blue), 0 mg $PO_4^{3-}$ $l^{-1}$ (light green), 25 mg $PO_4^{3-}$ $l^{-1}$ (purple), 50 mg $PO_4^{3-}$ $l^{-1}$ (pink), 200 mg $PO_4^{3-}$ $l^{-1}$ (orange), and 500 mg $PO_4^{3-}$ $l^{-1}$ (light blue).

Growth experiment on various levels of potassium.

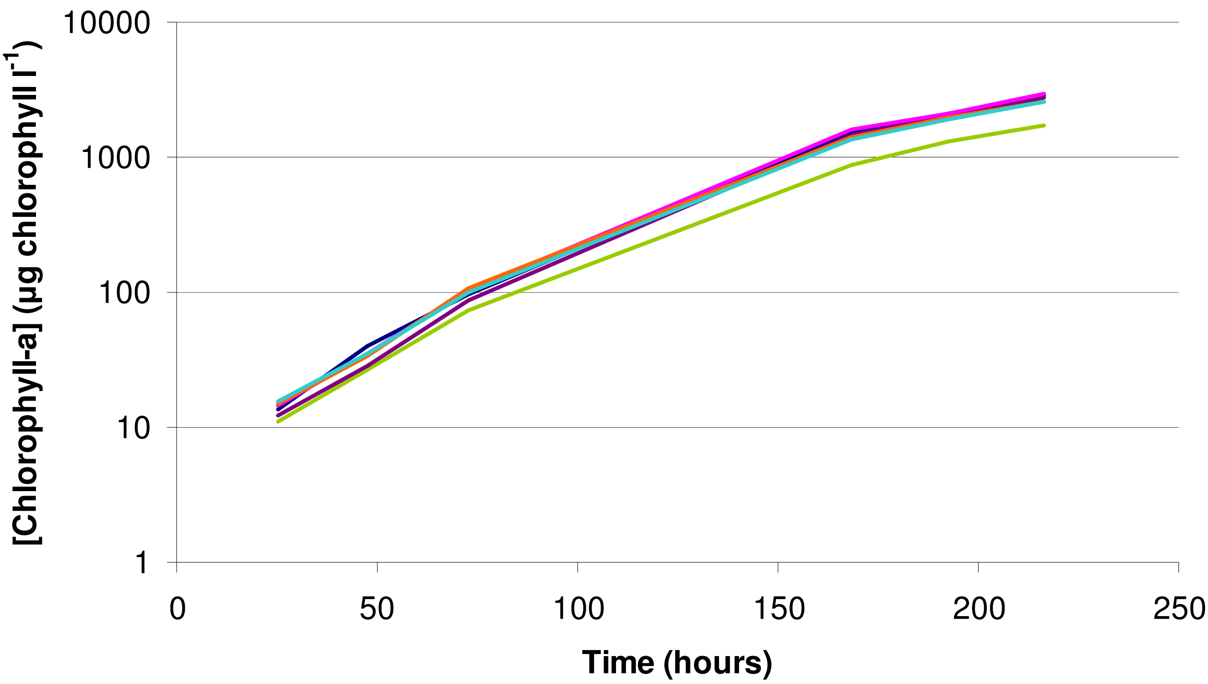


**Figure 3.1.** The mean chlorophyll-a concentration of *S. obliquus* plotted against time at different levels of potassium: WC medium (dark blue), 0 mg $K^+$ $l^{-1}$ (light green), 25 mg $K^+$ $l^{-1}$ (purple), 50 mg $K^+$ $l^{-1}$ (pink), 200 mg $K^+$ $l^{-1}$ (orange), and 500 mg $K^+$ $l^{-1}$ (light blue).

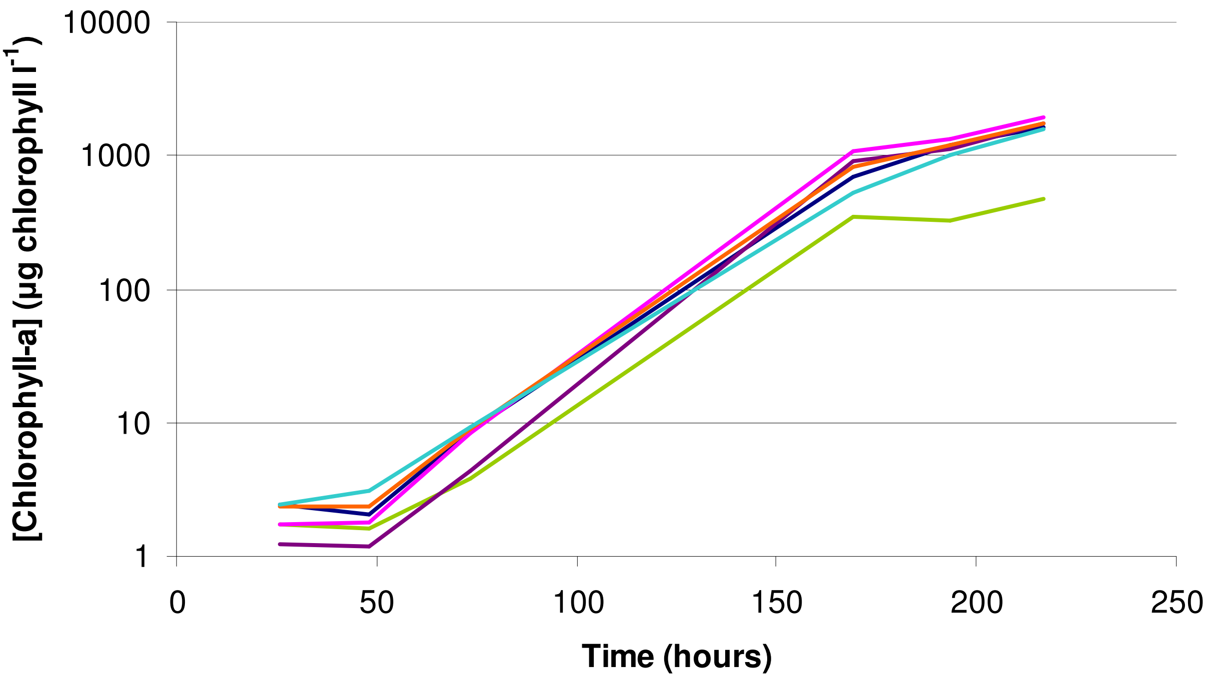


**Figure 3.2.** The mean chlorophyll-a concentration of *C. vulgaris* plotted against time at different levels of potassium: WC medium (dark blue), 0 mg $K^+$ $l^{-1}$ (light green), 25 mg $K^+$ $l^{-1}$ (purple), 50 mg $K^+$ $l^{-1}$ (pink), 200 mg $K^+$ $l^{-1}$ (orange), and 500 mg $K^+$ $l^{-1}$ (light blue).

<u>Growth experiment on various dilutions of digested pig manure.</u>

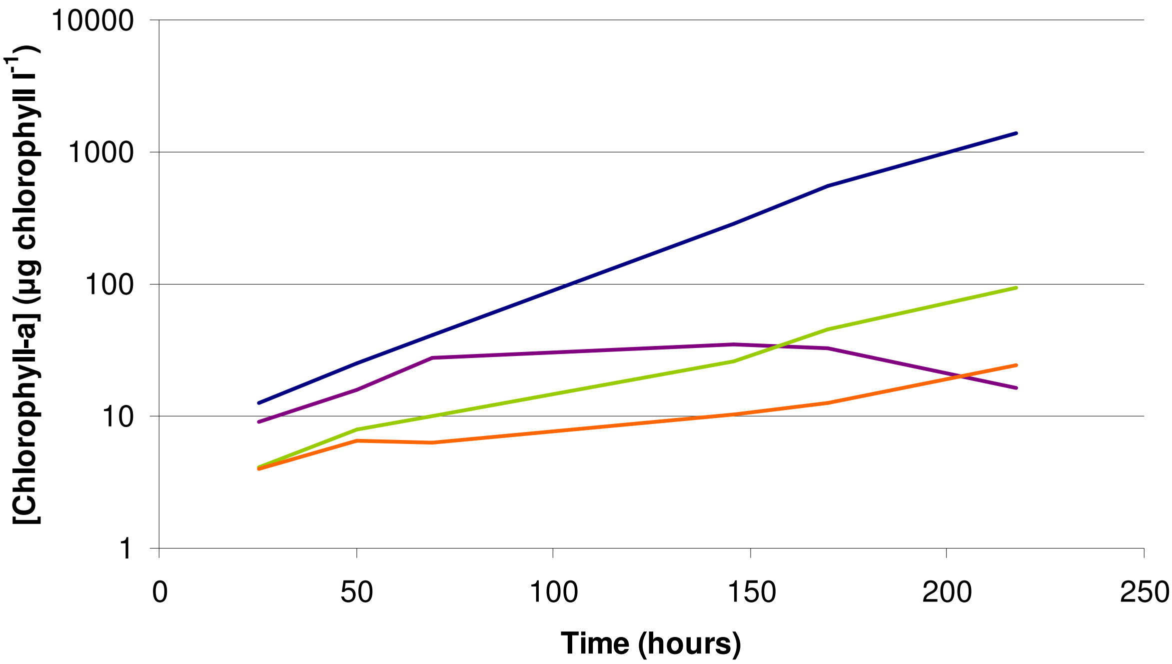


**Figure 4.1.** The mean chlorophyll-a concentration of *S. obliquus* plotted against time at different dilutions of digested pig manure: WC medium (dark blue), 0% digested pig manure (purple), 2.5% digested pig manure (light green), 5% digested pig manure (orange), 25% digested pig manure (pink), and 50% digested pig manure (brown).

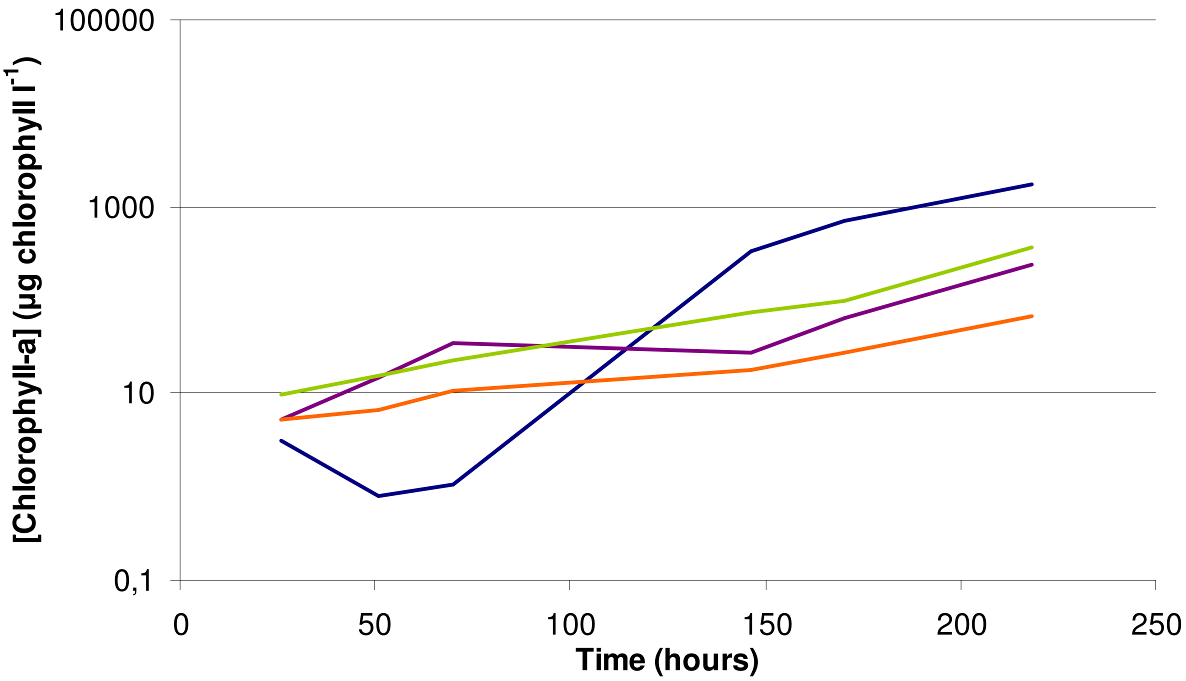


**Figure 4.2.** The mean chlorophyll-a concentration of *C. vulgaris* plotted against time at different dilutions of digested pig manure: WC medium (dark blue), 0% digested pig manure (purple), 2.5% digested pig manure (light green), 5% digested pig manure (orange), 25% digested pig manure (pink), and 50% digested pig manure (brown).

## Appendix 2 - Photosystem II efficiency (Yield) vs. Time

Growth experiment on various levels of ammonium.

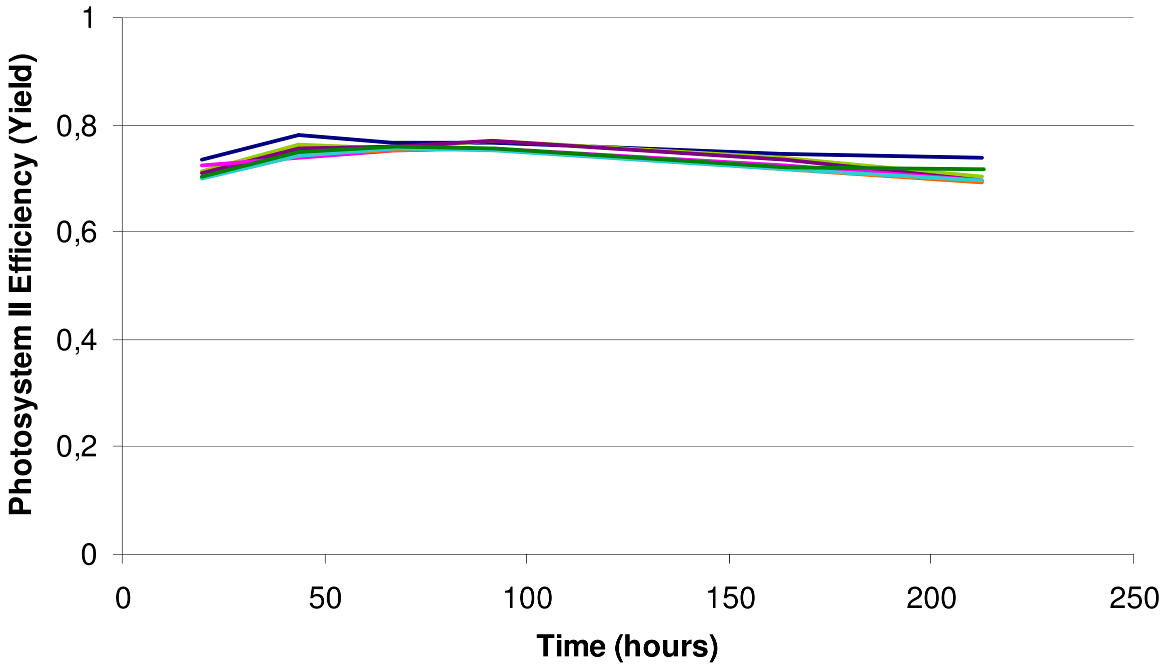


**Figure 1.1.** Photosystem II efficiency of *S. obliquus* plotted against time at different levels of ammonium: WC medium (dark blue), 100 mg $NH_4^+$ $l^{-1}$ (light green), 200 mg $NH_4^+$ $l^{-1}$ (purple), 400 mg $NH_4^+$ $l^{-1}$ (pink), 600 mg $NH_4^+$ $l^{-1}$ (orange), 800 mg $NH_4^+$ $l^{-1}$ (light blue), and 1000 mg $NH_4^+$ $l^{-1}$ (dark green).

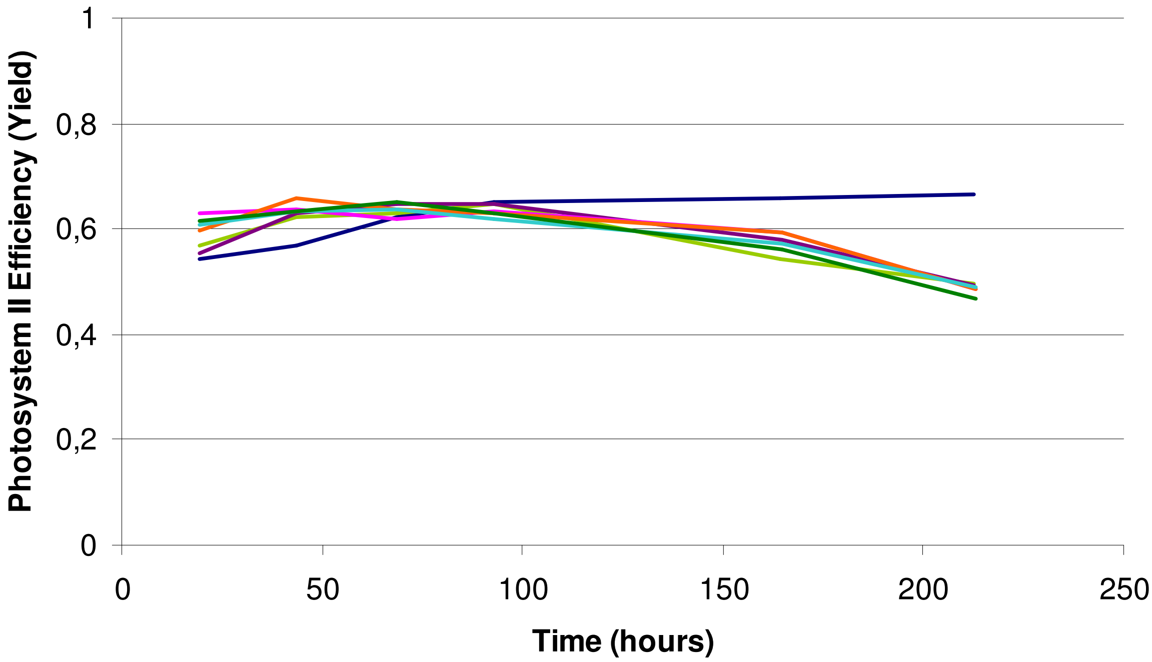


**Figure 1.2.** Photosystem II efficiency of *C. vulgaris* plotted against time at different levels of ammonium: WC medium (dark blue), 100 mg $NH_4^+$ $l^{-1}$ (light green), 200 mg $NH_4^+$ $l^{-1}$ (purple), 400 mg $NH_4^+$ $l^{-1}$ (pink), 600 mg $NH_4^+$ $l^{-1}$ (orange), 800 mg $NH_4^+$ $l^{-1}$ (light blue), and 1000 mg $NH_4^+$ $l^{-1}$ (dark green).

Growth experiment on various levels of phosphate.

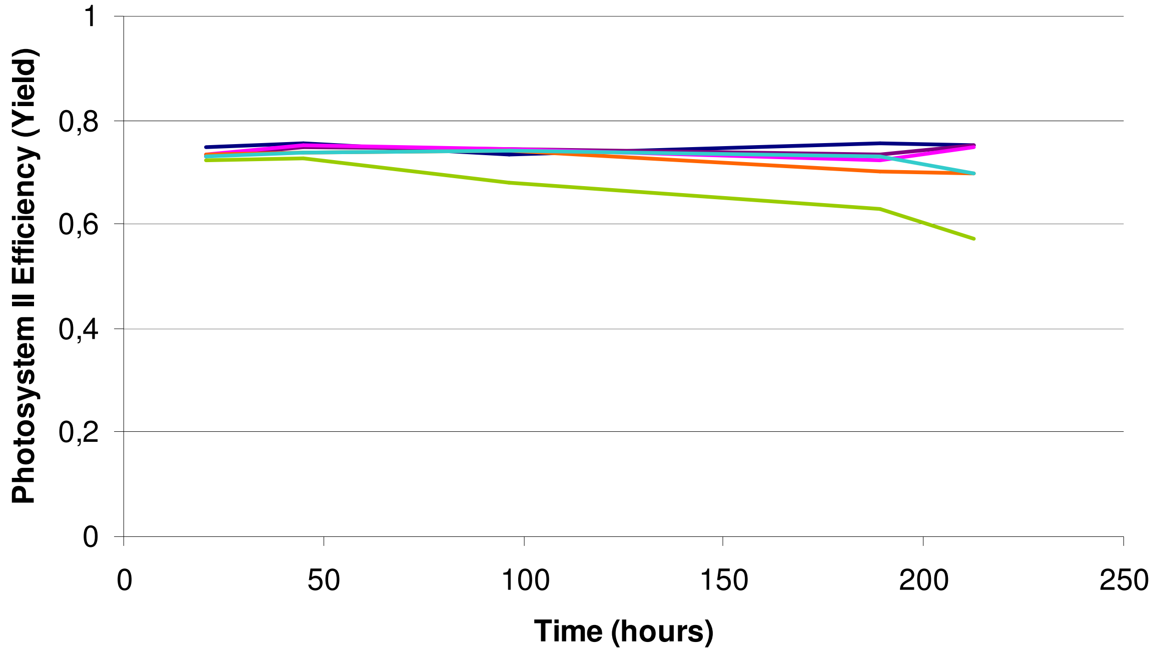


**Figure 2.1.** Photosystem II efficiency of *S. obliquus* plotted against time at different levels of phosphate: WC medium (dark blue), 0 mg $PO_4^{3-}$ $l^{-1}$ (light green), 25 mg $PO_4^{3-}$ $l^{-1}$ (purple), 50 mg $PO_4^{3-}$ $l^{-1}$ (pink), 200 mg $PO_4^{3-}$ $l^{-1}$ (orange), and 500 mg $PO_4^{3-}$ $l^{-1}$ (light blue).

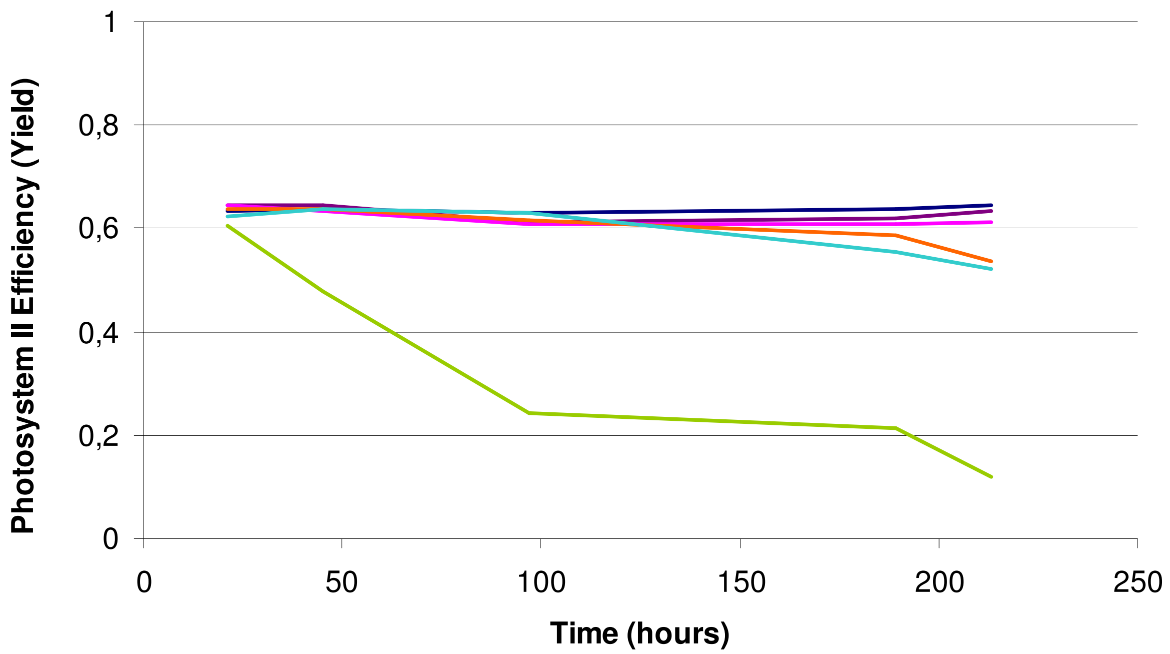


**Figure 2.2.** Photosystem II efficiency of *C. vulgaris* plotted against time at different levels of phosphate: WC medium (dark blue), 0 mg $PO_4^{3-}$ $l^{-1}$ (light green), 25 mg $PO_4^{3-}$ $l^{-1}$ (purple), 50 mg $PO_4^{3-}$ $l^{-1}$ (pink), 200 mg $PO_4^{3-}$ $l^{-1}$ (orange), and 500 mg $PO_4^{3-}$ $l^{-1}$ (light blue).

Growth experiment on various levels of potassium.

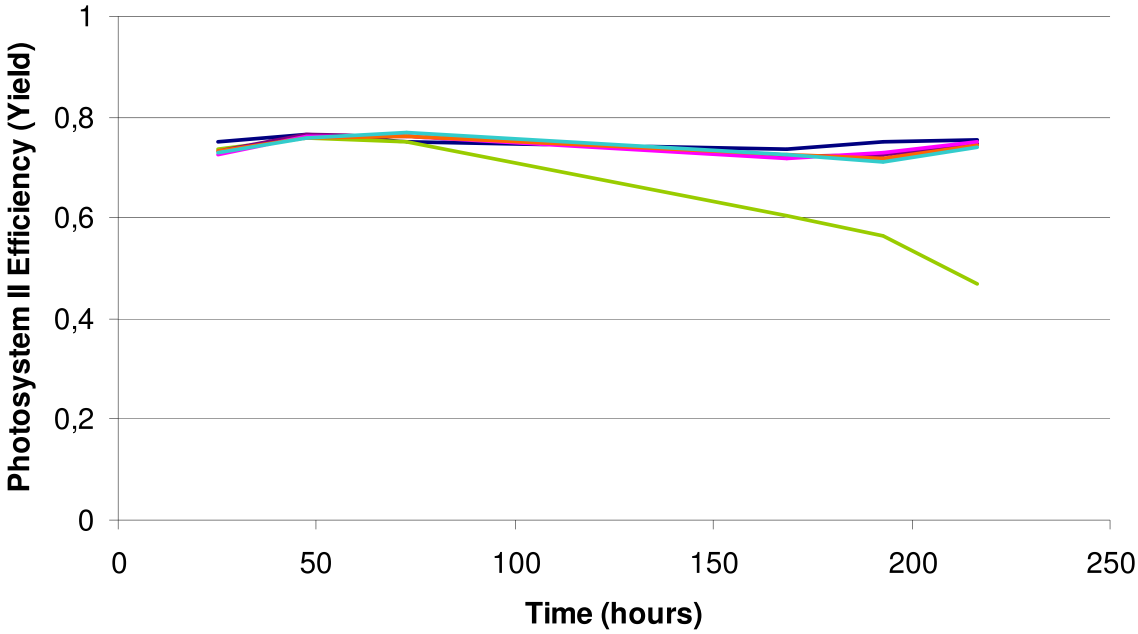


**Figure 3.1.** Photosystem II efficiency of *S. obliquus* plotted against time at different levels of potassium: WC medium (dark blue), 0 mg $K^+$ $l^{-1}$ (light green), 25 mg $K^+$ $l^{-1}$ (purple), 50 mg $K^+$ $l^{-1}$ (pink), 200 mg $K^+$ $l^{-1}$ (orange), and 500 mg $K^+$ $l^{-1}$ (light blue).

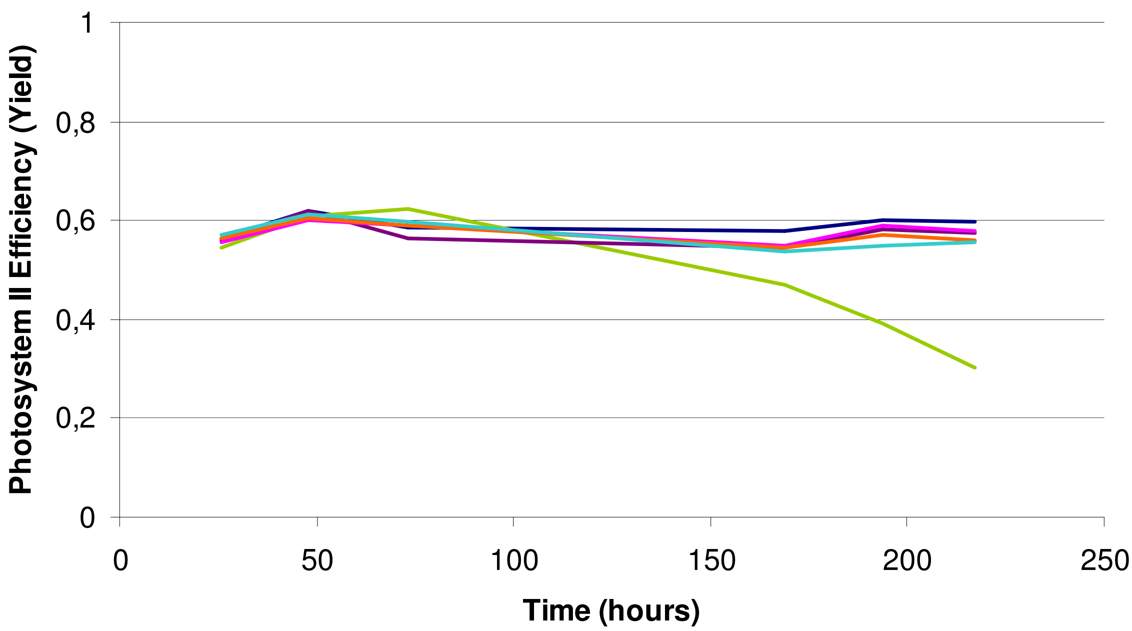


**Figure 3.2.** Photosystem II efficiency of *C. vulgaris* plotted against time at different levels of potassium: WC medium (dark blue), 0 mg $K^+$ $l^{-1}$ (light green), 25 mg $K^+$ $l^{-1}$ (purple), 50 mg $K^+$ $l^{-1}$ (pink), 200 mg $K^+$ $l^{-1}$ (orange), and 500 mg $K^+$ $l^{-1}$ (light blue).

Growth experiment on various dilutions of digested pig manure.

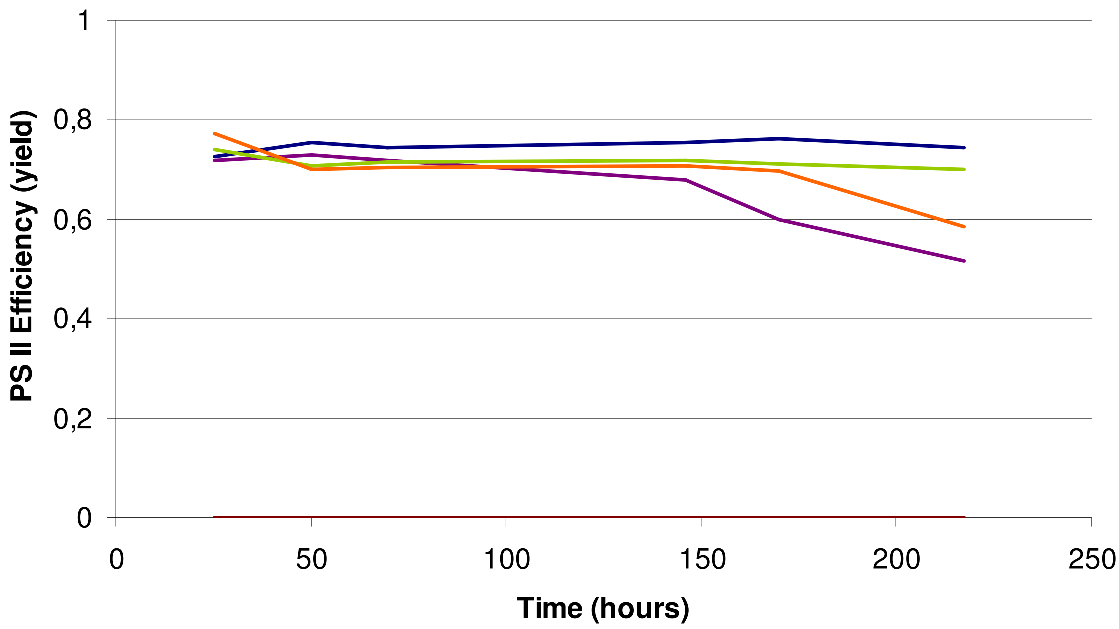


**Figure 4.1.** Photosystem II efficiency of *S. obliquus* plotted against time at different dilutions of digested pig manure: WC medium (dark blue), 0% digested pig manure (purple), 2.5% digested pig manure (light green), 5% digested pig manure (orange), 25% digested pig manure (pink), and 50% digested pig manure (brown).

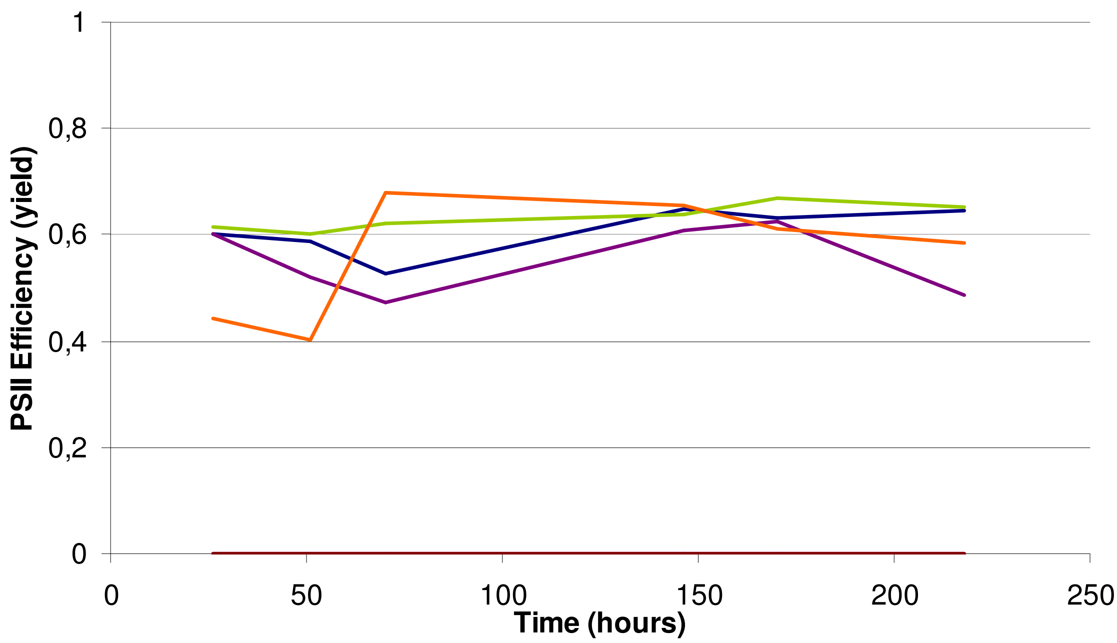


**Figure 4.2.** Photosystem II efficiency of *C. vulgaris* plotted against time at different dilutions of digested pig manure: WC medium (dark blue), 0% digested pig manure (purple), 2.5% digested pig manure (light green), 5% digested pig manure (orange), 25% digested pig manure (pink), and 50% digested pig manure (brown).

## Appendix 3 – Chlorophyll-a per µm³

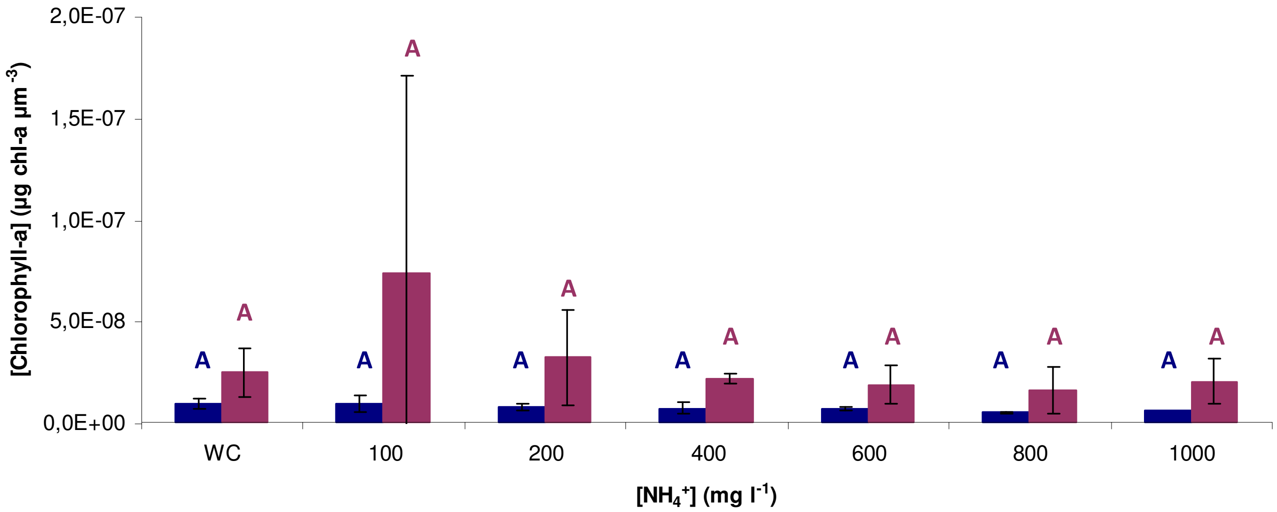


**Figure 1.** The amount of chlorophyll-a per µm³ for *S. obliquus* (blue) and *C. vulgaris* (pink) at different concentrations of ammonium, including the standard deviations. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous groups of yield rates of *C. vulgaris*.

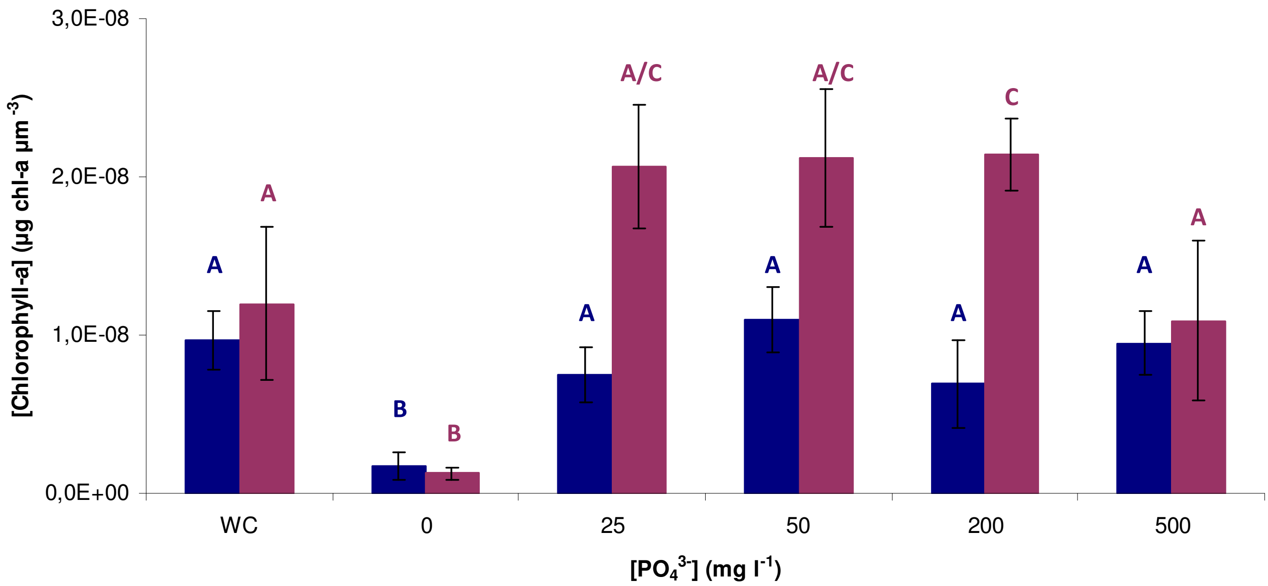


**Figure 2.** The amount of chlorophyll-a per µm³ for *S. obliquus* (blue) and *C. vulgaris* (pink) at different concentrations of phosphate, including the standard deviation. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous groups of yield rates of *C. vulgaris*.

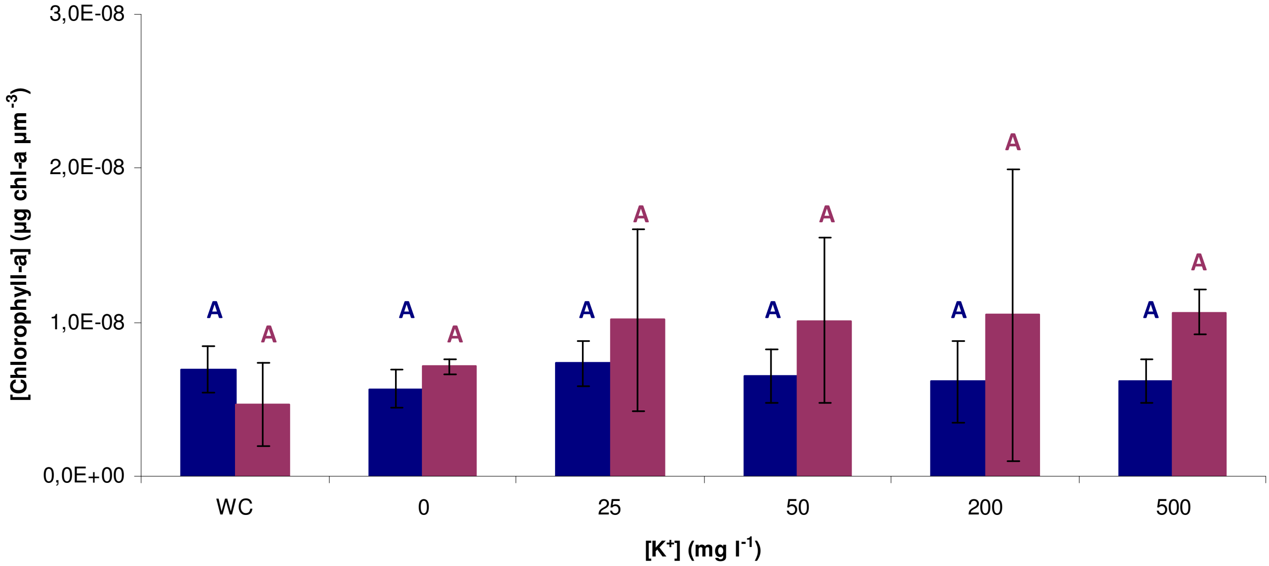


**Figure 3.** The amount of chlorophyll-a per µm$^3$ for *S. obliquus* (blue) and *C. vulgaris* (pink) at different concentrations of potassium, including the standard deviation. Blue capital letters represent homogeneous subsets of yield rates of *S. obliquus*, while pink capital letters represent the homogeneous groups of yield rates of *C. vulgaris*.