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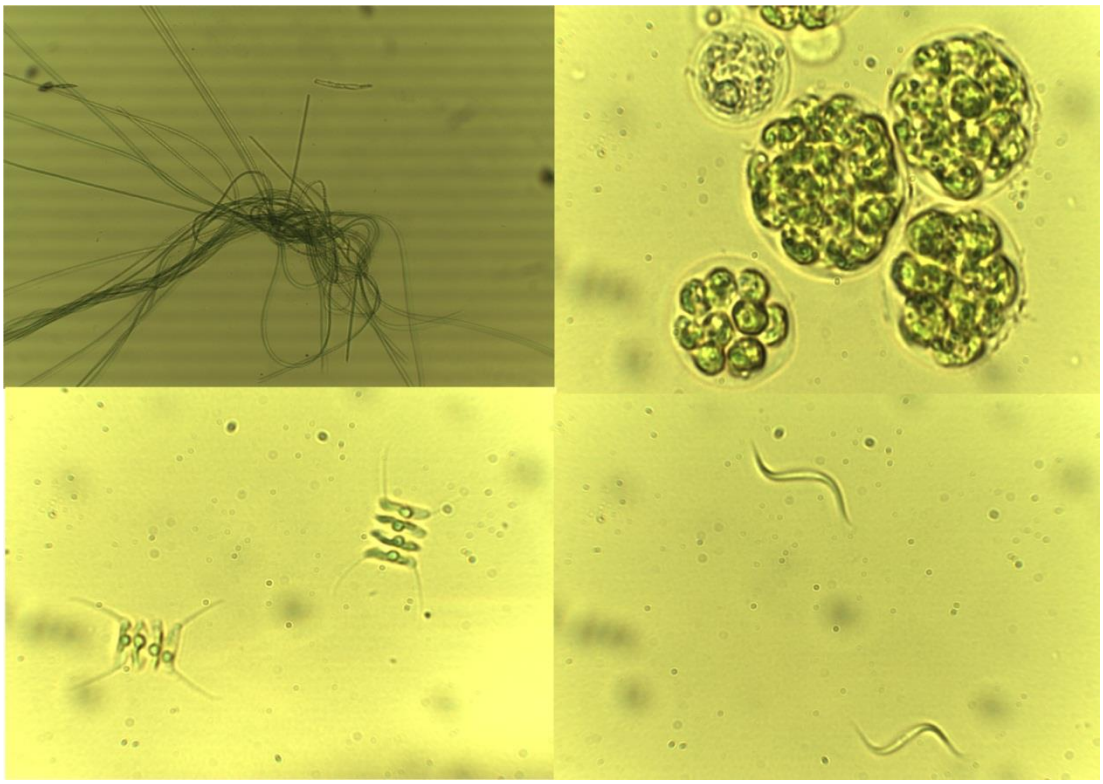

Improved utilization of waste resources from the pulp and paper mill *Nordic Paper Bäckhammar* and isolation of local freshwater microalgae species

Master of Science Thesis in the Master Degree Program Biotechnology

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Abstract

This master thesis was performed during a full academic year and aimed at investigating the possibilities in utilizing otherwise discarded biosludge as a source of nutrients for algal cultures. This work was done as a part of a project cofounded by Vinnova and ÅF in collaboration with the Swedish pulp and paper mill *Nordic Paper Bäckhammar* (NPB) and SP, *Technical Research Institute of Sweden (SP)*. Biosludge was chosen as a possible source as it is produced *in situ en masse* and contained a large mass fraction of nitrogen (2.8 % g/g). Biosludge was pre-treated with acids, bases and ultrasonication prior to experiments to determine an effective method for nutrient leaching. Acid pre-treatment showed promising results and was chosen as a suitable method for further development during this project. A screening was performed to evaluate the effect of heat and time versus leached nutrient concentrations from biosludge in HCl-solutions. The results indicated that a high temperature, 120 °C and a long reaction time, 24 h, reached the highest concentrations of NH_4^+ (19 mg/l) and PO_4^{3-} (60 mg/l). The highest concentration of NO_3^- was 64 mg/l, reached after 170 h at 25 °C. The volume was kept constant during all leaching experiments.

Water samples were collected at NPB for isolation of microalgae, which were cultivated and stored in minimal laboratory medium, 3N-BBM+V. Algae monocultures were isolated *via* serial dilution and plate streaking. Based on a visual examination, 7 different morphologies were found among the isolated cultures: *Phormidium sp, Oscillatoria sp, Coenococcum sp, Scenedesmus dimorphus, Scenedesmus quadricauda, Chlorella vulgaris* and *Monoraphidium contortum. S. quadricauda* and *C. vulgaris* could grow in treated biosludge-based medium. *C. vulgaris, S. dimorphus* and *M. contortum* showed fast growth rates in 3N-BBM+V medium and 15 % CO_2, with growth rates of 0.065, 0.064 and 0.054 h^{-1} respectively. The total lipid content were determined for the algae strains in selected conditions and it was found that *M. contortum* could contain as much as 45 % lipids with a fatty acid composition suitable for biofuel production.

Acknowledgement

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Content

1. Introduction

1.1. Background

One of society's greatest challenges is to reduce the environmental effects caused mainly by industrial activities, but also from our everyday life. Global warming has been extensively studied and a multitude of future scenarios have been proposed [3]. Countermeasures of different kinds have been implemented to reduce the atmospheric warming; some are economic, *e.g.* control of CO_2 [4], and some are scientific, *e.g.* CO_2 sinks or increased process efficiency for reduced emissions by [5]. All counteracting measures aim for one of two effects, either to reduce the amounts of greenhouse gases already present in the atmosphere or to diminish the amounts continually released.

Photoautotrophic organisms, such as terrestrial green plants, microalgae and photosynthetic bacteria, utilize CO_2 as a carbon source and light as energy source via photosynthesis. Previous studies have shown promising results regarding the possibility to grow microalgae in reactors using flue gas exhausts and other waste streams as nutrient sources to obtain a valuable biomass for production of different bio-based substances [6-8]. The produced biomass can then be processed and refined into a multitude of products, such as ethanol, methanol, hydrogen gas, diesel, pigments, etc. These products will grant a profit from the produced algae, thereby creating additional revenue [9-11]. Bio-based products can replace the production of chemicals and substances that today are produced in a petrochemical way and thereby add both economical and ecological value to the process. This project is intended to continue down that path, focusing on the development of a cheap but still effective process of recycling the carbon from exhaust gases into biomass while keeping it as inexpensive as possible. SP Technical Research Institute of Sweden is a key player in the microalgae field, leading a large three-year project (2013-2015) sponsored by Vinnova and ÅF at the Swedish pulp- and paper mill Nordic Paper Bäckhammar AB (NPB) and this thesis is an important part of this project. This thesis is a continuation of previous thesis work done by Appelberg [12] and Öberg [13].

To lower costs it is necessary to use whatever resources that are readily available within the specific industry. Solar energy can be used as a light source, flue gas as a carbon source, process- and wastewater for nutrients (mainly nitrogen and phosphorous), as well as waste heat [14, 15]. Nutrients are present not only in the water, but are also concentrated in the sludge produced in the sewage treatment process. The use of this produced sludge will be a central part of this project. Agricultural regulations restrict the use of sludge due to its often high heavy metal content, meaning it can't be used as a fertilizer despite the high content of nutrients. At *NPB*, the produced biosludge is only used as soil filler instead of effectively utilizing the nutrients [16]. This is one reason why this project is highly interesting. It would enable a new effective step in the sewage treatment process, as well as providing a free source of essential nutrients. For efficient algal cultivations the challenge is to develop a suitable leaching technique to satisfy the nutrient demand and also couple together the reactor with flue gas for CO_2 provision. The addition of CO_2 would enhance the algal growth and thereby increase the demand for available nutrients in the medium [17]. The medium should be clear for efficient light penetration and still provide sufficient levels of nutrients [18].

Another challenge when cultivating algae in an open system is the risk of contamination and thereby loss of the inoculated species and the aimed monoculture [19]. The species with the most effective growth will outgrow any other species present in the cultivation. Reasonably, these effective species should already be present in the local surroundings. It is costly to keep a monoculture in a bioreactor, and therefore it would be highly beneficial to use local species with interesting cell composition. Contamination may still occur, but will be minimized. A mixture of algae species could, however, be

beneficial if the consortium of algae has the right properties. Isolation, identification, and evaluation of local species that already thrive in the environment are therefore an important aspect of this project.

1.2. Aims

The central questions that will be investigated are:

1. Is it possible to use sludge as a nutrient source for freshwater microalgae?
2. How can nutrients be leached from sludge to the freshwater medium most effectively? What, if any, pre-treatments such as weak acid treatment or digestion of sludge can be utilized to improve the release of nutrients?
3. Which nutrient is the growth limiting factor? Is it possible to increase leaching of this nutrient?
4. Are there any local microalgae species of interest at NPB that can be used to fixate CO_2 while being suitable for production of any high-value bio-products?
5. Is it possible to cultivate any local algae isolates in biosludge-based growth medium?
6. Do the isolated microalgae have interesting cell contents of total proteins, total carbohydrates or total lipids?

1.3. Limitations

This study will use simulated flue gas instead of the actual flue gas produced at NPB due to logistical difficulties. The alga used for evaluating different leaching models of nutrients from sludge will be *Scenedesmus dimorphus* (UTEX 417) originally isolated in Lund, Sweden. This was the chosen inoculum strain for the first-year cultivation in 2013for the Bäckhammar Algae Farm pilot plant located at *NPB*. However, algal growth rate and biomass formation on biosludge should be compared to measured growth rates on nutrient rich growth media, thereby indicating the availability of leached nutrients and the effectiveness of the leaching process compared to commercially available alternatives. All performed experiments were of lab-scale.

2. Theory

2.1. Microalgae and microalgae cultivations

Microalgae is a diverse group of very different organisms, of marine and freshwater species, filamentous and solitary species, red, green and diatom species, motile and non-motile species *etc.* One common property is that all species can utilize phototrophic energy to survive and proliferate.

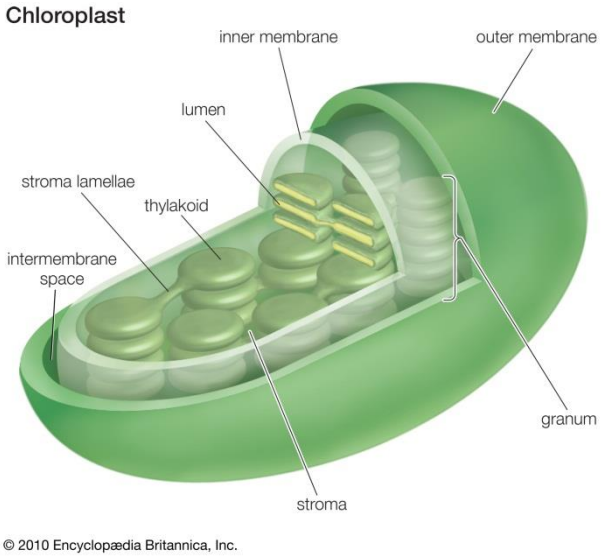


Figure 1: An overview of an algal chloroplast. Image taken from Encyclopædia Britannica Online 2014-04-28

2.1.1. Photosynthesis

Photosynthetic reactions occur in the chloroplasts within the cell. Inside the chloroplast are thylakoids, which contain all of the cells chlorophyll. The chloroplast can be seen in Figure 1. The photosynthetic energy reactions are separated in two sections, light-reactions and light independent reactions as described by O'Connor [20], and in two texts by Barsanti [21, 22]. In the light reactions H_2O is oxidized to O_2, releasing electrons in the process. These are then used to fixate CO_2 in the light independent reactions, and produce the molecules needed for cell survival, *e.g.* carbohydrates, proteins and fatty acids. An overview of these processes is shown in Figure 2 [20, 21].

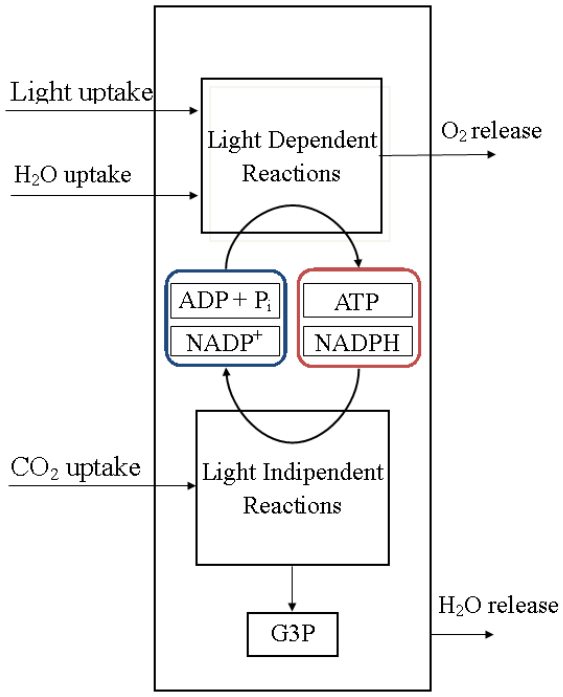


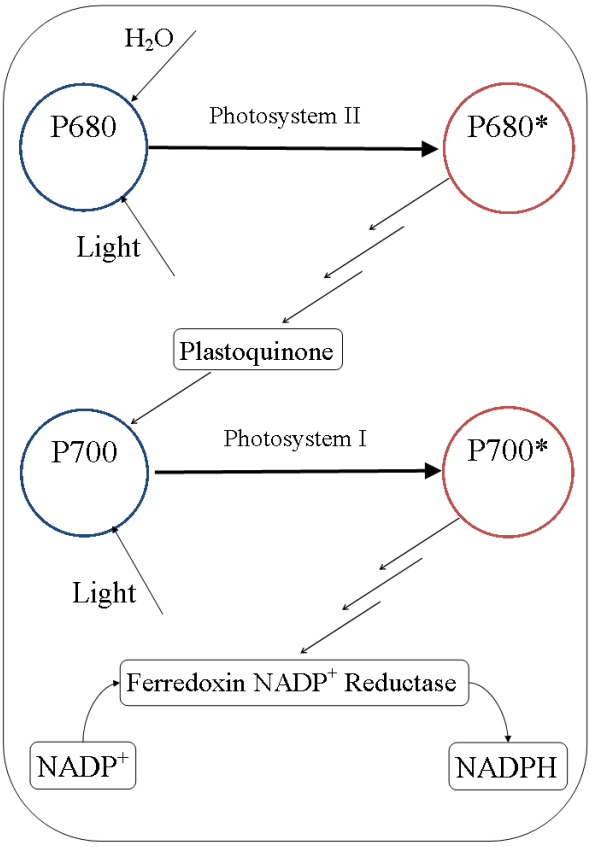
Figure 2: An overview of the photosynthetic processes, showing the two separate subsystems. Light and water is utilized in the light dependent reactions, generating ATP and NADH. These energetic molecules are consumed in the light independent reactions to form G3P, a substrate to form glucose.

Light reactions

Two major systems are involved in the light reactions, photosystem I (PS I) and photosystem II (PS II) shown in Figure 3 [22]. According to Barsanti [22], PS II is responsible for the formation of protons, electrons and O_2 from water via a light energy input. Light energy excites an electron in complex P_{680}, which is transferred via several protein intermediaries to plastoquinone. The now charged complex P_{680}^{+} is reduced in another reaction cascade that results in a charged $(Mn)_4$ cluster, which then oxidizes water molecules into oxygen and protons.

The total reaction of PS II is

$$H_2O \xrightarrow{Light\ energy} 2H^+ + 2e^- + \frac{1}{2}O_2$$



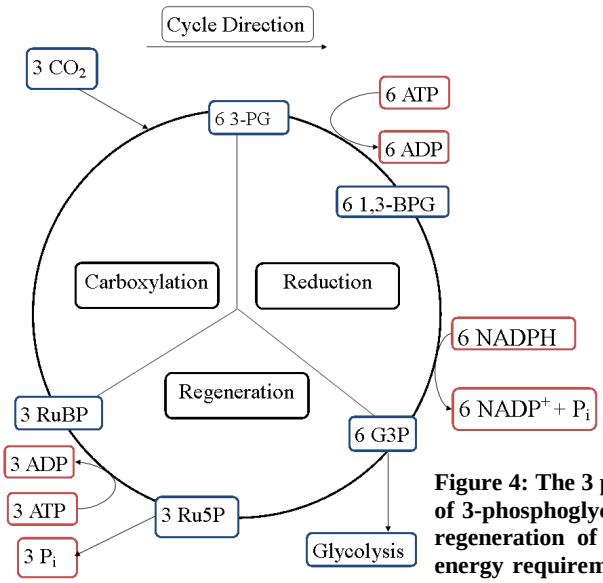
Barsanti [22] also describes how the protons form a pH-gradient across the thylakoid membrane. This leads to the production of ATP via an ATPase as protons flow to the stroma to equalize the gradient. The electrons carried from PSII by plastoquinone are used in PS I in a separate cascade reaction to generate NADPH via ferredoxin-$NADP^+$ reductase. A new photosensitive complex P_{700} is excited by light to generate electrons that are transferred to the stroma and ferredoxin via Fe_4S_2 clusters. Ferredoxin then provides $NADP^+$ reductase with electrons which in turn generates NADPH [22]. Both formed ATP and NADPH is used in the light independent reactions to bind CO_2 [1].

Figure 3: The light reactions of the photosynthetic process, here displayed in the "Z-scheme" layout. The light reactions are performed in two sections with two photosystems, PSI and PSII. PSII initiates the reaction by splitting water into oxygen, protons and electrons, and generating electrons to PSI, which utilizes a second light excitation to generate electrons to $NADP^+$ reductase and form NADPH.

The light reactions can be summarized with the formula:

$$4NADP^+ + 2H_2O + 4ADP + 4P_i \xrightarrow{8\ Photons\ +4e^-} 4NADPH + 4ATP + O_2$$

Light independent reactions



The light independent reactions are responsible for the biosynthesis of carbohydrates from CO_2, which is done via the Calvin cycle shown in Figure 4 [23]. The Calvin cycle reactions can be summarized into the following equation

$$3CO_2 + 9ATP + 6NADPH \rightarrow Glyceraldehyde\text{-}3\text{-}P + 9ADP + 8P_i + 6NADP^+$$

Figure 4: The 3 phases of the Calvin cycle; carboxylation of CO_2, reduction of 3-phosphoglyceric acid, 3-PG, to glyceraldehyde 3-phosphate, G3P, and regeneration of ribulose 1,5-biphosphate, RuBP. The process has great energy requirements in the form of ATP and NADPH, both of which are provided from the light reactions shown prior.

This process has high energy requirements, as 3ATP and 2NADPH is needed for every fixated CO_2-molecule [24]. Barsanti [22] describes the process as follows. The first step of CO_2 fixation is the reaction between CO_2 and ribulose 1,5-bisphosphate (RuBP) via the enzyme RuBisCO. The formed molecule is a 6C-molecule that is split into two separate identical 3C-molecules, 3-phosphoglyceric acid (3-PG). Each 3-PG is then reduced to glyceraldehyde 3-phosphate (G3P), a molecule usable in gluconeogenesis. This process consumes both an ATP as well as a NADPH molecule for each 3-PG. 2 G3P molecules were formed from the CO_2 fixation. One of which is used to form carbohydrates and one is used to regenerate the original CO_2 acceptor RuBP. The G3P molecule is converted to the intermediary Ru5P, which is then phosphorylated to RuBP by another ATP.

The light reaction systems and light independent reaction system cooperate to produce the necessary metabolites needed for CO_2 fixation. All energy originates from the sun and generates carbohydrates with the familiar equation for photosynthesis: [22]

$$6CO_2 + 18ATP + 12NADPH + 12H_2O \rightarrow C_6H_{12}O_6 + 18ADP + 18P_i + 12NADP^+ + 6H^+$$

2.1.2. Algae cultivation requirements

A functional cell requires many different elements to ensure cell survival [25, 26]. A list of all elements and the relative abundance in algae can be found in Table 1. According to Grobbelaar [25] carbon is utilized by either autotrophic (CO_2) or heterotrophic (organic compounds) growth, or possibly a mix of the two (mixotrophic growth) [27]. Different species are capable of different growth modes, *e.g. S. quadricauda* can grow mixotrophically on both organic carbon as well as CO_2 [28], whereas *P. tricornutum* only can utilize autotrophic growth [29].

Table 1: A list of nutrients and the relative cell composition in algae. List taken from [25] 2014-04-28

A list of nutrients required by algal cells

Element	*Cell composition µg/mg dry weight*
C	175-650
O	205-330
H	29-100
N	10-140
Na	0-4-47
K	1-75
Ca	0-80
P	0.5-33
S	1.5-1.6
Mg	0.5-75
Fe	0.2-34
Zn	0.005-1
Mn	0.02-0.24
Si	0-230

Nitrogen is often the growth limiting nutrient in a culture and a great substantial nitrogen supply is required for substantial growth [30]. Nitrogen can be supplied in both inorganic forms such as NH_4^+, NO_2^-, NO_3^- *etc.* or organic substances such as urea, amino acids, pyrines, *etc.* It will be present as several of these compounds when using natural sources, *e.g.* waste water, as a source for cultivation media [17, 25].

Fabricated media have the benefit of exactly known compositions, but are expensive to manufacture in greater quantities. A natural nutrient source, *e.g.* waste water is often very inexpensive but might be sub-optimal for use as growth medium, due to a dark coloration, toxic concentrations of various substances or suboptimal levels of needed elements.

Algae are phototrophic organisms and are thus able to utilize light as an energy source. The so called Photosynthetic Active Radiation, or PAR for short, are photons with wavelengths between 400-700 nm, which can be used in photosynthetic processes [2]. A limited light influx in the culture will limit algal growth [31]. However, heavy light stimulation might also be harmful. This might cause a loss of photoreceptors and degrade chlorophyll, while also stimulating the production of photo-protective carotenoids zeaxanthin and β-carotene. It is therefore necessary to illuminate algae cultures with enough light for photosynthesis to function, but not enough to cause photo inhibition [31, 32]. The effect of the light influx on the specific growth rate can be viewed in Figure 5.

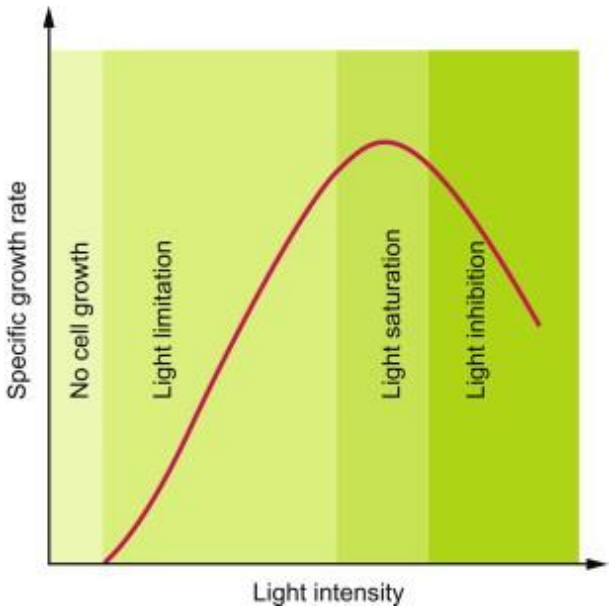


Figure 5: The correlation between light intensity and specific growth rate in an otherwise uninhibited system.

pH in growth media

All organisms are more or less sensitive to environmental pH-changes. Freshwater microalgae are known to thrive in somewhat neutral pH. [33, 34]. However, reports have also shown that some freshwater alga, *e.g. Scenedesmus dimorphus*, have been successfully cultivated in acid media with pH ~3 [28]. The pH can be controlled in several ways; as described by Richmond [35], it tends to rise as an algal culture grows on carbon, but is lowered if CO_2 is introduced to the medium by carbonic acid formulation. This is because of the bicarbonate buffering system shown below that is formed between CO_2 and H_2O.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

This buffer shows a connection between the solubility of CO_2 in water and the pH; at low pH the equation is driven to the left due to an abundance of H^+ ions, whereas at high pH the opposite happens. This is described by Chen *et al* [36] where they evaluate the growth of different species in higher pH (up to pH 9). They conclude that CO_2 availability becomes the primary growth limiting factor at high pH.

2.2. Isolation techniques for microalgae

Suitable techniques for isolating cells vary with the cell type. Algae are commonly separated with one or several of the following techniques; micromanipulation, serial dilution and streak plating. [37, 38]

Micromanipulation is a method where an operator manually chooses and transfers a single cell from a sample to a new sterile medium with a micropipette in a microscope. It is then grown until a monoculture can be confirmed via microscopy. It is an exact technique, but highly time-consuming. The new growth is slow, due to the origin from a single cell, but at the same time it is possible to be highly selective in which cells are transferred to form pure colonies.

Serial dilution is the use of multiple containers with sterile medium, where a small sample volume is transferred to a container with sterile medium, which is stirred thoroughly. A small volume is drawn and transferred to another container with sterile medium and stirred etc. By doing this enough the original sample is diluted until only a single cell is transferred and results in a pure monoculture. This technique is faster and easier to perform since only sterile containers and medium is needed. Drawbacks include the risk of transferring two or more cells to a sterile medium and thereafter not transferring any cells to the next container. This would not yield a single monoculture but a mixture of several species. Another drawback with this technique is that only one colony can be purified from each serial dilution when it might be necessary to isolate a multitude of different cells from the original sample.

Streak plating can be and often is used in combination with a serial dilution. Plates are filled with a sterile nutrition medium with an addition of a gelation agent, typically agar. This creates a semi solid gel that contains all nutrients present in the original medium. However, introduced cells will be stationary at the surface of the gel. A small volume of liquid containing cells is placed on the gel and streaked out over the surface, hence the name streak plating. If this liquid contains a high cell number the resulting colonies will possibly cover the entire plate and selection of individual colonies will be all but impossible. It is therefore beneficial to combine the serial dilution with the streak plating, to find an optimal dilution where there are several colonies to transfer but no trans-colonial growth is observed. These cells will divide and grow by themselves, resulting in pure colonies which may be transferred to new containers. This technique requires more preparations than only a serial dilution since the agar gel has to be made. But, beneficially it is possible to choose exactly which colonies that should be transferred. This will only work with cells capable of growing stationary on a surface.

2.3. Freshwater microalgae species

Algae are a diverse group of organisms that differs greatly between species. They show great morphological variability as well as different environmental requirements for optimal growth. This work will focus on a few freshwater species isolated in NPB, with morphological information taken from John *et al* [39]. The name of each species and strain is based on morphology.

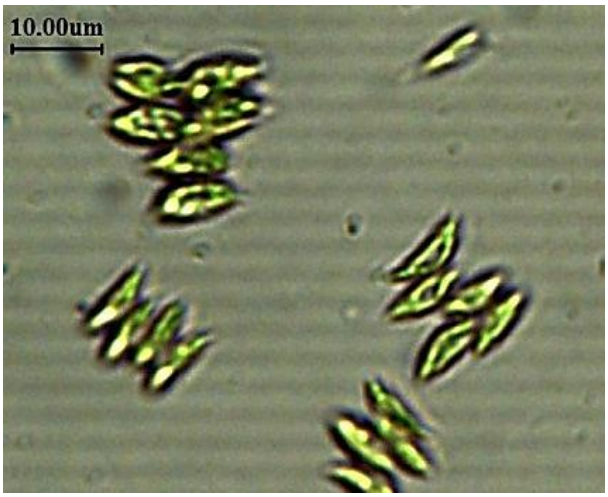


Figure 6: ***Scenedesmus dimorphus*****, 400x**

2.3.1. Scenedesmus dimorphus

S. dimorphus grows both unicellularly and in groups of 2, 4 and 8 cells. It is a common cosmopolitan species that is typically twice as long as it is wide (2 – 9.4 µm wide and 6 – 25 µm long) and is broadly spindle shaped. *S. dimorphus* is effective in nutrient uptake from surrounding medium and has been shown to effectively reduce nutrient levels, making it an interesting species for waste water purification [40]. Lipid content has been reported at 34 %, after the cultures were subjected to nitrogen starvation [41].

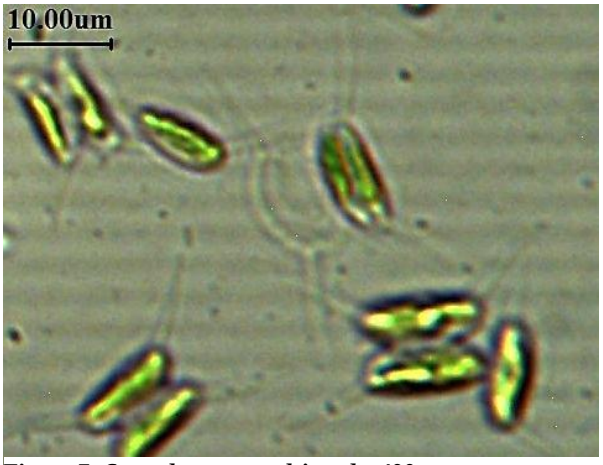


Figure 7: ***Scenedesmus quadricauda*****, 400x**

2.3.2. Scenedesmus quadricauda

S. quadricauda is commonly found solitary or in groups of 2-4, linearly arranged cells, with four spines protruding from the two outer cells. Cells are about 3x longer than wide (9 – 17 µm long and 3.5 – 6.6 µm wide). This species is cosmopolitan. Cells can utilize mixotrophic growth and studies have shown that this affects the resulting lipid content, which has been recorded at 33.1 % w/w of the biomass [42].

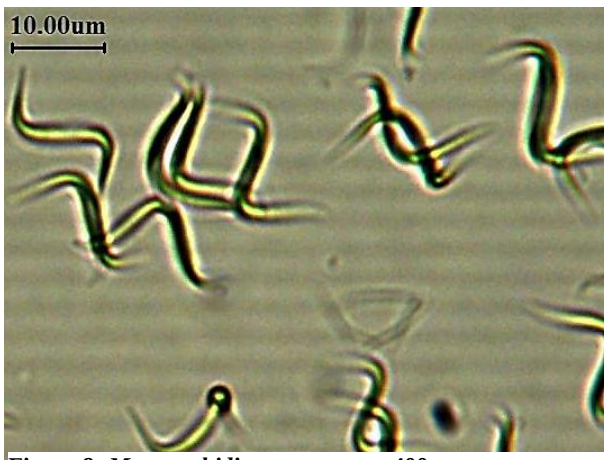


Figure 8: ***Monoraphidium contortum*****, 400x**

2.3.3. Monoraphidium contortum

M. contortum is a cosmopolitan species that is long and needle-like, possibly narrowly spindle shaped. Cells are 7 – 40 µm long but only 1-2.2 µm wide and often spirally twisted. *Monoraphidium* is considered a promising alga for biodiesel production due to a high reported lipid content (32.9 % w/w) and fast growth rates [43]. *Monoraphidium* has also been shown to have high uptake rates regarding N and P, which also makes the alga interesting in waste water treatments [44].

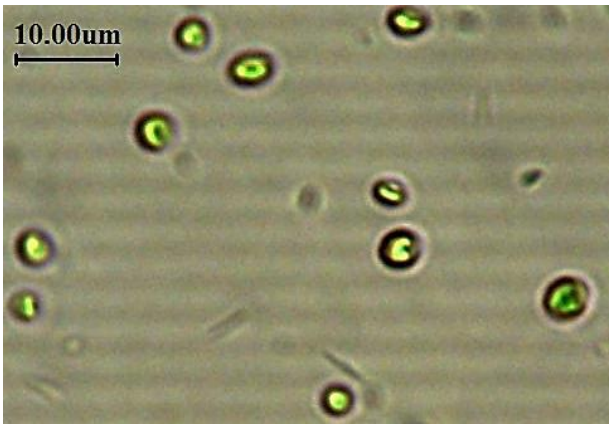


Figure 9: ***Chlorella vulgaris*****, 400x**

2.3.4. Chlorella vulgaris

Chlorella species are very similar to each other, all being spherical cells and might be difficult to classify. *Chlorella vulgaris* is 1.5 – 10 µm in diameter and probably cosmopolitan. Experiments have shown that *C. vulgaris* has a high lipid yield (> 30% w/w biomass) [45] and a fast biomass production when cultured in waste water [46].

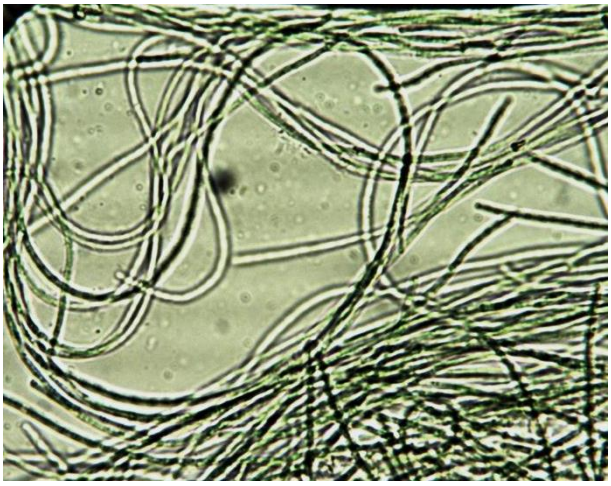


Figure 10: ***Phormidium sp*****, 400x**

2.3.5. Phormidium sp

This species is difficult to determine morphologically. There are many species in the *Phormidium* family and they are found in both wet soil as well as fresh and brackish water. This is a filamentous alga which forms large mats on surfaces. *Phormidium* reportedly has a good nutrient uptake rate from waste water. Coupled with the natural flocculation of *Phormidium* this makes it a promising species for waste water treatment purposes [47].

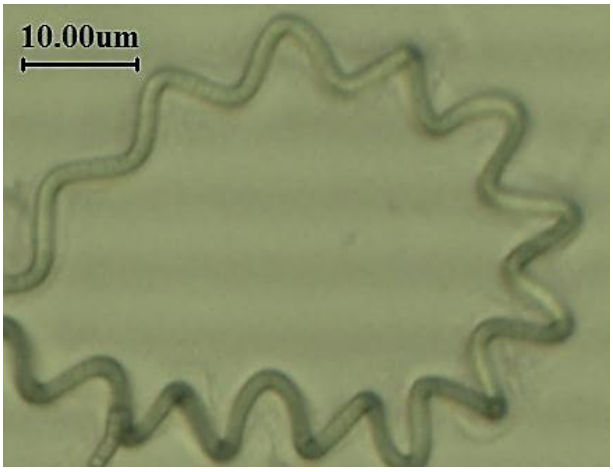


Figure 11: ***Oscillatoria sp*****, 400x**

2.3.6. Oscillatoria sp

Oscillatoria is a motile, filamentous algae family, containing several species. One specie, *O. agardhii* is a toxic algae, producing hepatotoxic compounds and is problematic in algal bloom [48]. *O. limnetica* is capable of utilizing anoxygenic photosynthesis, using Na_2S as an electron donor instead of O_2 [49]. *Oscillatoria* does not appear to be part of any studies involving waste water treatment or biofuel production.

2.4. An overview of a typical waste water treatment process

Waste water treatment processes are similar whether the water has industrial or domestic origins. A simplified overview can be found in Figure 12. The released quantity of organic matter and nutrients must be controlled to avoid eutrophication of recipient waters [50, 51]. These processes are performed in several phases that vary depending on the water type, but commonly included are mechanical filtering, chemical and biological treatments and flocculation, sedimentation and removal of sludge [52, 53].

First mechanical filters sort out fragments from the water, often in several steps with gradually finer meshes. This is to separate and remove larger fragments as well as protect machinery such as pumps from clogging [54].

Chemical treatments are based on aggregation of nutrient salts and sediment formation which can be easily separated from the water. One nutrient that is commonly removed by chemical treatments is phosphorous, which forms aggregates in the presence of some metallic cations, *e.g.* iron and aluminium [53]. Phosphorous and nitrogen are the two main nutrients responsible for eutrophication and should be fully removed [55, 56].

Removal of other nutrient salts, mainly nitrogen compounds, requires other methods than chemical treatments. This is often performed by a biological treatment with bacteria during a two-step process, nitrification and de-nitrification [57]. Ammonia is first reduced to nitrite and nitrate (nitrification), which is then further reduced to nitrogen gas (de-nitrification) which goes to the atmosphere. The organic matter present in the liquid is simultaneously reduced during this phase since the bacteria are using available carbon together with dissolved nitrogen compounds [57, 58].

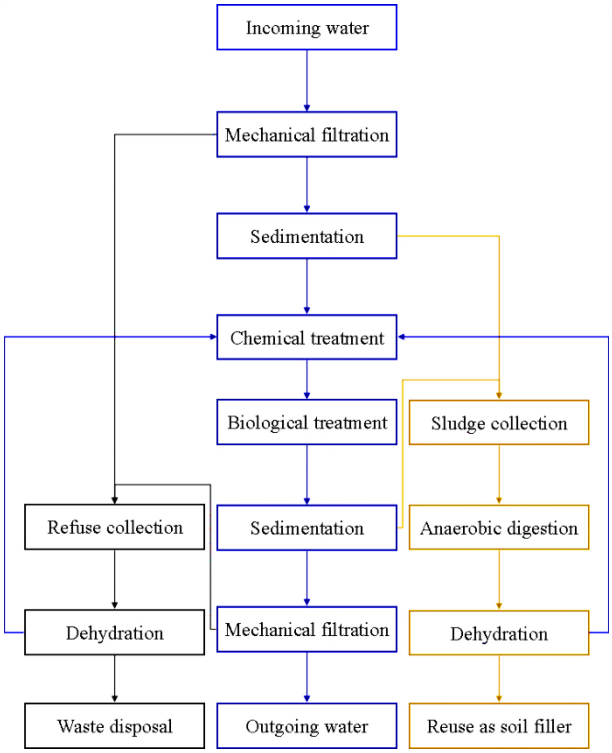


Figure 12: A step-by-step overview of the treatment process of waste water. Black borders for refuse processes, blue for water processes and brown for sludge processes. For example: The second step in the process is a mechanical filtration step. Water and larger objects are separated by a mesh. The water goes through to the sedimentation while the separated objects are dehydrated and later collected for disposal. The dehydrated water goes back into the process once again.

It is important that the bacterial culture thrives fully to achieve optimal purification. If the content of organic matter, nitrogen and phosphorous is unbalanced it might be necessary to add missing nutrients to achieve a maximum growth rate. *e.g.* if the incoming water has a high amount of organic matter, but is deprived of other nutrients it might be necessary to add those to achieve unlimited growth. The same argument is valid if the water has a high content of nitrogen compounds, but is meager in organic carbon or phosphorous [56, 58].

Small objects that passed the mechanical filtration together with aggregates from the chemical treatment, bacteria, and degradation products from the biological treatment are bound together by an added polymer. This helps the formation of a biosludge that is concentrated, collected, and removed

from the water [59]. The water can then be released to a recipient or undergo further treatments to reduce nutrients and particles even more, such as microfiltration [60]. The sludge has limited uses as of now since it often contains large amounts of heavy metals and other toxins, but this is highly dependent on the water origin. However, it also contains many nutrients and is as such a potential fertilizer. The use of biosludge is prohibited for most commercial areas of interest due to the often high toxic content which for example prevents any agricultural uses. [56, 60].

2.5. Pre-treatments of biosludge from the Bäckhammar mill

According to Svensen [16] at NPB most of the nutrients in the biosludge are suspected to be embedded in microorganisms. The sludge produced at NPB consists of mainly cellulosic fibers, lignin, microorganisms and precipitation agents such as aluminium sulphate and polyacrylamide. NPB produces approximately 20 metric tons of biosludge daily, which is a huge potential source for an inexpensive production of algae growth medium. To access the nutrients, cellular membranes need to be disrupted and lysed, or the fibers constituting the sludge to be disintegrated and thus free trapped nutrients, which is possible via several methods. Three of these methods were chosen for this project; acid-, base- and ultrasonic treatment. Ultrasonic treatments cause cavitations in the medium, which propagates through the sample. These cavities can form extreme temperatures (>5000 K) and pressure (>1000 atm) which cause permeable gaps in the cellular membranes and release the cytosolic structures within [61, 62]. This might be effective in a small scale environment, but as volumes grow larger this technique becomes more expensive and less efficient [62].

pH can be effectively manipulated at a low cost, especially if acid and/or base can be found *in situ.* A strong acid or base will introduce highly reactive H^+ and OH^- ions that react with organic matter. Cellulose is difficult to hydrolyze due to incredibly large interactive forces within each fiber as well as between different fibers [63]. These reactions will degrade and lysate cells and cellulose fibers, which will enhance the nutrient availability. Cells that might otherwise be shielded by fibers will instead be exposed to the treatments [64].

2.6. Total lipid content and fatty acid profile assay

To evaluate the total content of lipids in cell biomass an assay is performed by lysing the cells and then phase separating polar cell structures *e.g.* proteins, from nonpolar structures, *e.g.* lipids. The separated nonpolar phase then contains all fatty acids together with glycerol in the form of tri-glycerides. It is possible to break the bonds between the fatty acids and the glycerol by methanolysis, after which another phase separation can be performed to purify pure fatty acids in a non-polar solvent [65, 66]. The method used in this project is a modified Folch method, which can be found in Appendix I.

2.7. Analytical methods

A variety of different analytical methods can be used to observe cells and determine growth.

2.7.1. Cell concentration determination

Two methods were used to follow growth, both manual cell count and optical density, OD measurements, both described by Sorokin *et al* [67]. A manual cell count is a direct measurement of the number of cells present in a sample, while OD-measurements are an indirect method that shows trends and differences between two samples and not the specific cell count. This is often adequate information as it is sufficient to follow the total culture growth in optical density and not exact cell numbers. The cell count is performed under a microscope with a Bürker chamber. The chamber has an etched grid that yields specific volumes of sample within the grid itself.

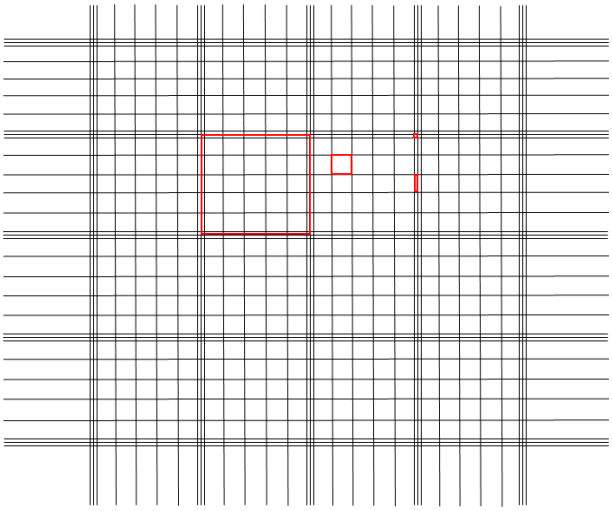


Figure 13: The etched grid of a cell counting chamber, where different square sizes have been highlighted in red. A glass cover slip is placed over the grid and liquid is injected between, forming a specified volume.

A grid used for calculating cell numbers is shown in Figure 13. In order to statistically ensure a true estimation of the cell count a number of squares have to be measured and averaged. The size of the chosen square depends on the observed cellular concentration, which makes it possible to use a counting grid for multiple concentrations. The use of a Bürkerchamber gives a direct estimation of the cell count in a sample, but requires a lot of manual work and preparations.

Optical density (OD), or turbidity, is measured with a spectrophotometer. The sample is placed in a cuvette and a blank sample zeroes the background absorbance by the medium without the measured component (*e.g.* algal cells). For each measurement, a lamp emits light with either one specific wavelength or a full spectrum and the absorbance is calculated via the logarithmic formula

$$A = -log_{10}\left(\frac{I}{I_0}\right)$$

Where A is the absorbance, I is the transmitted light and I_0 is the incident light, as shown in Figure 14. OD is a fast and easy technique, but gives an indirect indication of the biomass concentration in the sample. The obtained value is given as absorbance but the actual measure is the *diffraction* of light by the cells and not the sample's ability to *absorb* the light. Therefore it is important to minimize the errors by light harvesting pigments within the algae cells and use wavelengths where absorbance minimums exist. At low OD-values, around 0.2 and 0.5, the cell concentration is proportional to the OD, but

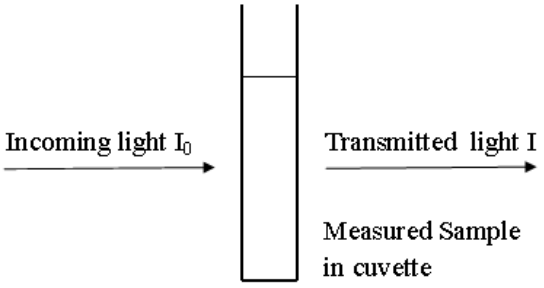


Figure 14: The theory of optical density measurments. A lamp emits chosen wavelengths and the transmitted light is measured.

at higher values the cells start to shade each other and dilutions are necessary. It does not account for changes in the sample matrix; therefore it is not a suitable method if the component of interest produces substances that might affect the measured OD, such as chlorophylls and other light harvesting pigments.

Griffiths et al [68] showed that OD_{750} was preferred, as an absorbance minimum by the chlorophylls and pigments was found at just 750 nm. Thereby the possible errors by absorbance differences were minimized. Pigments can vary within the microalgae by growth phase, time and environmental factors.

2.7.2. CHN analysis theory

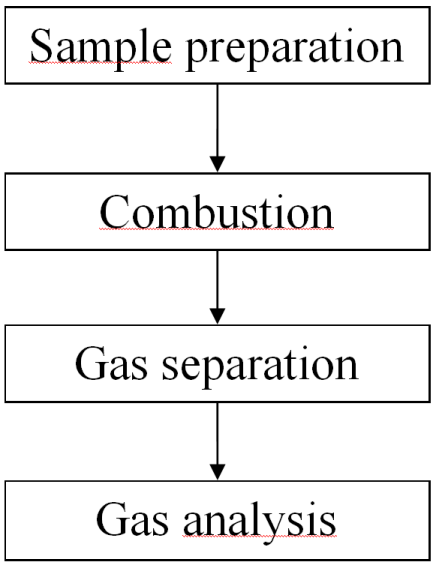


Figure 15: The layout for a typical elemental analysis. The sample is homogenized and dried in the preparation step, and then combusted in a combustion chamber.

CHN analysis is a major technique among the elemental analyses. A dry, homogenous sample is combusted under high temperature (1000 °C) and a continuous excess flow of oxygen to form gaseous oxides of all available carbon, hydrogen and nitrogen.

$$C \rightarrow CO_2$$

$$H \rightarrow H_2O$$

$$N \rightarrow NO_x \rightarrow N_2$$

These gases are then separated from any remaining solids and analyzed with an IR-detector to investigate the respective amounts of CO_2 and H_2O. NO_x-gases are reduced to N_2 when in contact with Cu, which can be measured with a TC-cell. [69]

3. Materials and Methods

This work was based on 3 major experiments. First, the growth of the alga *S. dimorphus* directly on biosludge dissolved in water was evaluated in 5l glass reactors. Secondly, the improved release of nutrients from biosludge was evaluated by analysis of released nutrients and thirdly, several local algae species found *in situ* at NPB were subjected to several growth screening cultivations with varying growth media and high CO_2 supply.

3.1. Growth of *S. dimorphus UTEX 417* on biosludge in 5l glass reactors

Sludge was collected at NPB *2013-10-14* and stored at -40 ^{o}C. It was thawed and dried prior to each experiment. Nitrogen content was used as a comparative baseline between the growth media. All reactor tanks were to theoretically contain equal amounts of total nitrogen, as in 3N-BBM+V medium, (124 mg N/l), thus, yield a total of 620 mg N in a reactor with 5 l working volume. Data from NPB coupled with results from an elemental analysis at SP, Borås suggested that approximately 25 g biosludge was sufficient to reach a level of 620 mg N. For detailed calculations, see Appendix II.

3.1.1. Experimental setup

Glass reactor tanks with 5 l growth medium were placed on magnetic stirrers under Growth Technology T5HO 4x24 W lamps with a light/dark cycle of 16/8 hours. The photon flux was measured at 90 µmol·m^{-2}·s^{-1}. Compressed air with no added CO_2 was fed and divided to the 8 reactors at a total feed rate of 1 l/min. The liquid temperature was 25-30 °C. The reactors were placed in room temperature, but were also slightly heated by the magnetic stirrers. 50 ml blank sample was drawn and stored before algal inoculation. pH and OD_{750} were measured daily.

The equipment used is shown in Figure 16 and shows the 8 reactors used. Tubing for inflow and outflow of gas is visible over each reactor, together with a particle filter for the inflow. Syringes for manual sampling were attached to a nozzle on each reactor.

3.1.2. Growth screening 1 of *S. dimorphus UTEX 417* on treated biosludge

Figure 16: The first screening setup. The photo is taken at the start of the experiment, just after inoculation. Biosludge is visible in nets in all reactors but the two on the right, being the two positive controls with NPK and 3N-BBM+V media instead. Notice the yellow discoloration in all reactors containing biosludge compared to reactor 7 and 8 to the right.

Biosludge screening 1 investigated algal growth on direct use of pre-treated biosludge added to the culture. This was compared to positive controls with a commercial fertilizer (NPK Tg Växupp, Hammenhögs) as well as 3N-BBM+V medium. Each reactor content and choice of pre-treatment method can be found in Table 2. Setups were done as singles in order to test a multitude of different pre-treatments and growth was measured for a total period over 20 days.

Resulting growth effects of a pure increase in sludge volume were investigated since additional sludge, larger surface area, acid and ultrasonic pre-treatments should yield additional nutrients compared to a smaller amount of untreated biosludge. Biosludge was loosened by manually pulling the fibers apart to create a more penetrable surface. Notice the discoloration of the water from the biosludge vs. the two controls in Figure 16.

*S. dimorphus UTEX 417 (*The University of Texas, http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=2937*)* was used as inoculum in all eight reactors and growth indicates if nutrients become available or not. 50 ml algal culture was centrifuged at 1100 xg for 5 minutes and the supernatant was discarded. The pellets were resuspended in 10 ml sterile water and added to each reactor. Initial pH was adjusted to 7 by addition of 1 M NaOH.

Table 2: The first screening of algae growth on biosludge. Five different methods of utilizing biosludge in nets were compared to a negative control of a small amount of biosludge as well as two positive controls of NPK and 3N-BBM+V media.

Evaluated biosludge pre-treatments of S. dimorphus UTEX 417 on treated biosludge, screening 1

Reactor #	*Content*
1	25 g Biosludge in Net
2	100 g Biosludge in Net
3	4x25 g Biosludge in Nets
4	100 g Loosened Biosludge in Net
5	100 g 10 % H_2SO_4 Pre-treated Biosludge in Net
6	100 g Ultra sonic Pre-treated Biosludge in Net
7	Positive Control 1 (4.4 g NPK in 5 l Deionized Water)
8	Positive Control 2 (5 l 3N+BBM+V Medium)

3.1.3. Growth screening 2 of *S. dimorphus UTEX 417* on biosludge

Figure 17: The second growth screening setup. The media are from left to right: NPK-control, biosludge dissolved in water, biosludge dissolved in HCl and biosludge dissolved in NaOH. This photo is taken at the end of the experiment, after 3 weeks growth. No biosludge fibers were present in the reactors as only the filtrate from the pre-treatments was introduced. Notice the resulting differences in coloration between the reactors. Medium based on biosludge dissolved in NaOH (to the right) was much darker than the rest, resulting in the darkest culture. This must not be confused with a greater algae population as cell density was evaluated by OD_{750}.

Biosludge screening 2 investigated the effects of a more thorough pre-treatment by liquefying the dried biosludge in either H_2O, in an acid or in a base. The remaining fibers were filtered and removed with Munktell 3 filters and the remaining liquid was adjusted to pH 7. In biosludge screening 2 duplicate cultures were used.

NPK was chosen over 3N-BBM+V as the positive control due to ease of preparations and no observable differences in resulting growth were found in growth screening 1. Acid pretreatment of biosludge was performed with 10 % HCl. Alkaline pre-treatment of biosludge was performed with 10 % NaOH. High pH is excellent for disrupting cells and this should lysate all microorganisms within the sludge, providing all available nutrients from them. A table of all nutrient media can be found in Table 3

50 ml culture of *S. dimorphus UTEX 417* was centrifuged at 1100 xg for 5 minutes and the supernatant was discarded. The pellets were resuspended in 10 ml sterile water and added to each reactor. Growth was followed by OD_{750} and measured daily during 13 days. Initial pH was adjusted to 7 in all reactors with the addition of 1 M NaOH or 1 M HCl.

Table 3: The second screening of algae growth on biosludge. Three different setups were evaluated with double samples and compared to a positive control of NPK-medium.

Evaluated biosludge pre-treatments of S. dimorphus UTEX 417 on treated biosludge, screening 2

Reactor #	*Content*
1	Positive Control 1 (4.4 g NPK in 5 l Deionized Water)
2	Positive Control 2 (4.4 g NPK in 5 l Deionized Water)
3	Filtrate From 25 g Biosludge 1
4	Filtrate From 25 g Biosludge 2
5	Filtrate From 25 g 10 % HCl Pre-treated Biosludge 1
6	Filtrate From 25 g 10 % HCl Pre-treated Biosludge 2
7	Filtrate From 25 g 10 % NaOH Pre-treated Biosludge 1
8	Filtrate From 25 g 10 % NaOH Pre-treated Biosludge 2

3.1.4. Acid, alkaline and ultra-sonic pre-treatments of biosludge

Biosludge was pre-treated to increase the availability of the nutrients. Acid, alkali and ultra-sonic conditions were used in conjunction with heat to disrupt cellular membranes and lysate cells contained in the sludge, while also separating nutrients trapped within the cellulosic fibers.

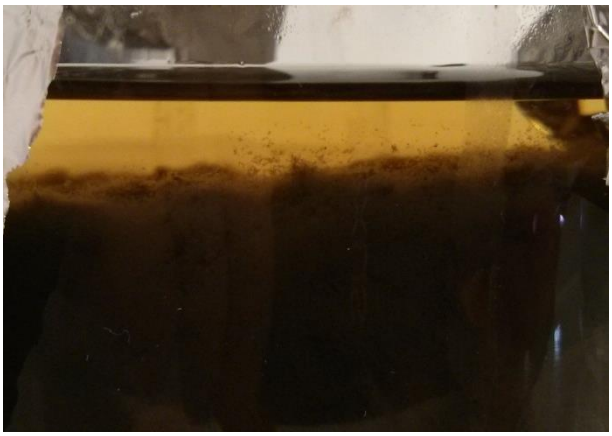


Figure 18: The image is taken from a quick test where 10 g biosludge was mixed with 100 ml 10 % HCl to study the effects of stirring versus non-stirring on biosludge solubility in acid. It was found to discolor a 10 % HCl solution with a yellow tone. The sludge is visible as the dark fibers underneath a clear liquid phase of HCl. The same effect was observed for other acids, such as H_2SO_4, but to a lesser extent. Stirred samples were more thoroughly fragmented and dissolved than non-stirred samples. Data not shown.

Acid pre-treatment was tested with two different acids, sulphuric acid (H_2SO_4) and hydrochloric acid (HCl). H_2SO_4 was chosen since it is already being used at NPB as a pH control in the waste water treatment process. HCl was selected due to its highly oxidative nature.

H_2SO_4 pre-treatment was performed as follows: 200 ml 5 % H_2SO_4 was added to 100 g biosludge and heated at 120 ^oC for 1 hour in sealed PTFE-chambers followed by 24 hours of cooling at room temperature. PTFE is an inert plastic and was used to hinder damage on laboratory equipment by containing the formed corrosive gases. The fluid and intact fiber residues were pH-adjusted with 1 M NaOH to pH 7 and the fluid was added to the reactor tank. Appropriate amounts of pre-treated biosludge fibers were then collected in plastic nets and attached inside each reactor, which then were filled with deionized water to a total volume of 5 l and autoclaved at 121 ^oC for 20 min.

HCl pre-treatment was performed as follows: 200 ml 10 % HCl was added to each 25 g biosludge sample. The fibers were dissolved in the acid to yield a somewhat homogenous fluid which was heated to 120 ^oC for 1 hour in sealed PTFE-chambers and cooled for 24 hours. The liquid was adjusted to pH 7 with 1 M NaOH and filtered through Whatman glass microfiber filters grade GF/C with 1.2 µm pores. The filtrate was added to the reactor, and filled to a total volume of 5 l with deionized water and autoclaved at 121 ^oC for 20 min. The remaining sludge on the filter was discarded after the filtration.

Alkali pre-treatment was performed with 200 ml 1 M NaOH instead of acid, otherwise carried out as HCl pre-treatment described prior.

For the ultrasonic pre-treatment 200 ml deionized water was added to 100 g biosludge placed in a plastic net and placed in a Bandelin Sonorex Super RK 1028H ultrasonic bath for 30 min. The liquid and netted fibers were then transferred to the reactor tank where deionized water was added to a total volume of 5 l. The tank was then autoclaved at 121 °C for 20 min.

3.1.5. Formulation of positive control media 3N-BBM+V and NPK

Three different growth media were used in the experiments performed in this project, Bolds basal medium with added vitamins and 3x nitrogen, (henceforth called 3N-BBM+V or just Bolds), NPK-medium (henceforth called NPK) and treated biosludge medium. Bolds medium is often used for algal lab cultivation and is formulated to contain all necessary ingredients for viable growth. The nitrogen source of Bold's consists only of nitrate. NPK is a commercial fertilizer consisting of 14% nitrogen (N), 3% phosphorus (P), 15% potassium (K) and 10% sulfur (S). The nitrogen in NPK is 7.8 % NH_4^+ and 6.2 % NO_3^-, *i.e.* mainly ammonium. 3N-BBM+V and NPK medium recipes are found in Appendix III and IV.

3.2. Nutrient leaching measurements from HCl-treated biosludge

The second part of this work was to review nutrient leaching from pre-treated biosludge. A screening was performed to evaluate the impact of time and temperature on nutrient leaching in acidified biosludge. It was reasoned that a higher temperature and longer time would result in greater nutrient concentrations. This experiment was performed to yield an indication of the possibilities to perform an acid treatment at temperatures attained by waste heat produced at NPB,.

3.2.1. Experimental setup

0.5 g biosludge was placed in a PTFE-chamber with 50 ml 10% HCl. Time and temperature were varied and nutrients (NH_4^+, NO_3^-, and PO_4^{3-}) were measured with colorimetic assays by Hach-Lange cuvette tests for each component (analysis kits LCK339, LCK340 and LCK350). Each run was performed with triplicate samples. Each sample was filtered through a coarse Munktell 3 grade filter, the filtrate was collected and pH adjusted to 7. The filtrate was used for the nutrient analysis with the cuvette tests and measured with a Varian 50 biospectrometer at different wavelengths according to each respective protocol found in Appendix V, NH_4^+ was measured at 694 nm, NO_3^- was measured at 370 nm and PO_4^{3-} was measured at 850 nm. Absorbances were translated to concentrations via standard curves found in Appendix VI, from standard solution with known concentrations of 40 mg/l NH_4^+, 100 mg/l NO_3^- and 60 mg/l PO_4^{3-}, which were appropriately diluted.

Table 4: Design of leaching measurements from acidified biosludge. The different combinations of heat and time are presented below in a partial 3^2-factorial design. Room temperature (25 °C) was tested with 1, 24, 48 and 170 h, while higher temperatures (70 and 120 °C) were tested at 1 and 24 h.

3^2-factorial design			
Run	***Comb.***	***Factors***	
		Temp C	***Time h***
1	*neg*	25	1
2	*a*	70	1
3	*A*	120	1
4	*b*	25	24
5	*ab*	70	24
6	*Ab*	120	24
7	*B*	25	48
8	*aB*	25	170

The studied temperatures were room temperature (~25 °C), 70 °C and 120°C. Times were chosen to 1 h and 24 h for all temperatures, and also 48 h and 1 week (~170 h) for samples in room temperature. The full experimental design can be found in Table 4.

3.3. Sampling, isolation and growth rate screenings of isolated local species

The third part of this project was to isolate and evaluate growth rate differences and cell composition between any local species found *in situ* at NPB. A small-scale batch screening setup was developed to investigate the effects of high CO_2 exposure levels up to 15 % v/v. Increased CO_2 levels reduces carbon limitations for cell growth, but organisms might be sensitive to higher CO_2 levels, which would deem them unsuitable for use in flue gas cultures.

3.3.1. Sampling and isolation of local microalgal strains

Water samples were collected *in situ* at NPB in and around the existing pilot plant, *i.e.* from a raceway system, and Tethys reactor pools. Samples were also taken from the waste water treatment plant at NPB, from sedimentation ponds and bioreactors 1 and 2, as well as from the air by exposing a flask of 3N-BBM+V medium to the open surroundings. The samples were transferred to tissue culture flasks containing 20 ml 3N-BBM+V. The bottles were illuminated with 90 µmol photons/m^2·s during a 16/8 h light cycle for 2 weeks until algal growth was observed. 1 % agar plates were made from 3N-BBM+V medium and used for the isolation of individual cells to monocultures. The cultures were serially diluted up to 10^{10} x and 200 µl of each dilution was spread on agar plates, which also were placed under light with a 16/8 h light/dark cycle for 2 more weeks. Separate colonies were selected from the plates and transferred to 5 ml sterile 3N-BBM+V medium in 50 ml tissue culture flasks. The flasks were placed under light and later verified to be monocultures based on the algal morphology. An additional 35 ml new medium was added after monocultures were verified. Confirmed monocultures were inoculated in several back-ups for storage purposes. All flasks were examined regularly for infections and kept sterile.

3.3.2. Experimental setup

Six isolated monocultures were evaluated for growth rate in screening cultures with controlled gas flow using a gas mixer constructed by Johan Engelbrektsson, SP. Gas flow is controlled by separate regulators for each gas component. It is possible to monitor and change gas composition and flow rate on the fly by computer software. A total flow rate of 1.4 l/min with a gradually increasing CO_2 content from 0.04 % to 15 % was used for this experiment. CO_2 was increased stepwise to avoid a massive pH-drop by formed carbonic acid and to allow cells to acclimatise to the changed conditions. CO_2 increments were based on the work by Bark [70] and can be seen in Figure 19.

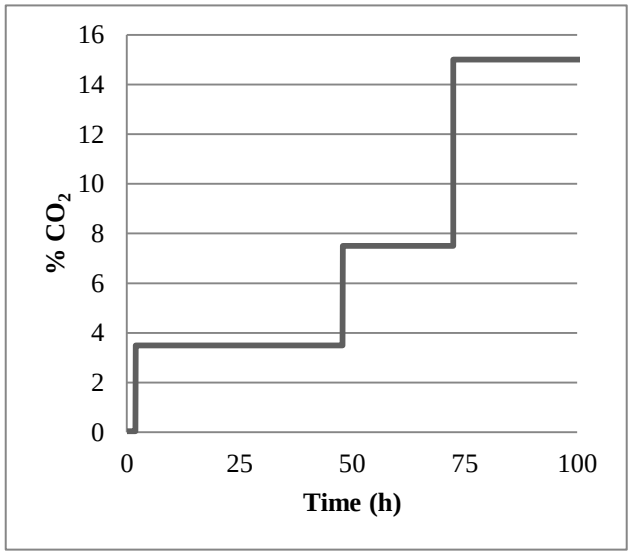
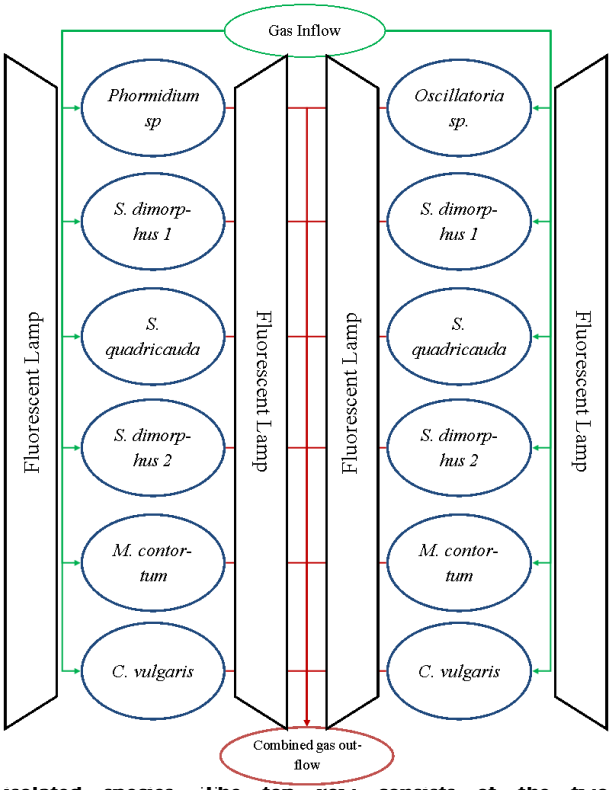


Figure 19: CO_2 % increments over time used in the growth rate screening of isolated species. The total gas flow rate was kept at a constant 1.4 l/min during the entire experiment.

The screenings were performed in 250 ml Erlenmeyer flasks. Flasks were positioned in two parallell rows with fluorescent lights around them illuminating all vessels in a 16/8 light/dark cycle. No mixing, by *e.g.* stirring, was implemented except that which was created by the gas bubbling. Samples for pH and OD_{750} measurements were collected every weekday for the entire cultivation period. Samples for lipid analysis were collected at the end of the period. Culture illumination was measured at 110 µmole photons /m^2/s.

3.3.3. Growth screening 1 of isolated local species on 3N-BBM+V medium



isolated species. The top row consists of the two filamentous species in singles, all other are duplicates. Two fluorescent lamps are placed next to each row of reactors illuminating from both sides in a 16/8 light/dark cycle.

Five isolates were chosen for this screening. The two filamentous species; *Phormidium sp* and *Oscillatoria sp*, and four free living species; *S. quadricauda*, *M. contortum*, *C. vulgaris* and *S. dimorphus*. The previously inoculated strain of *S. dimorphus UTEX 417* in the algal ponds at NPB was also included in this screening. The two filamentous species were cultivated in single cultures each, while the others were done in duplicates. The species were grown in 3N-BBM+V medium to evaluate any negative effects from a high CO_2 influx.

OD_{750} was measured in the selected algal cultures prior to inoculation to calculate suitable dilutions to attain similar initial OD_{750} values. The placements and a schematic view of the setup are shown in Figure 20. Photos of the screening setup can be seen in Figure 21.

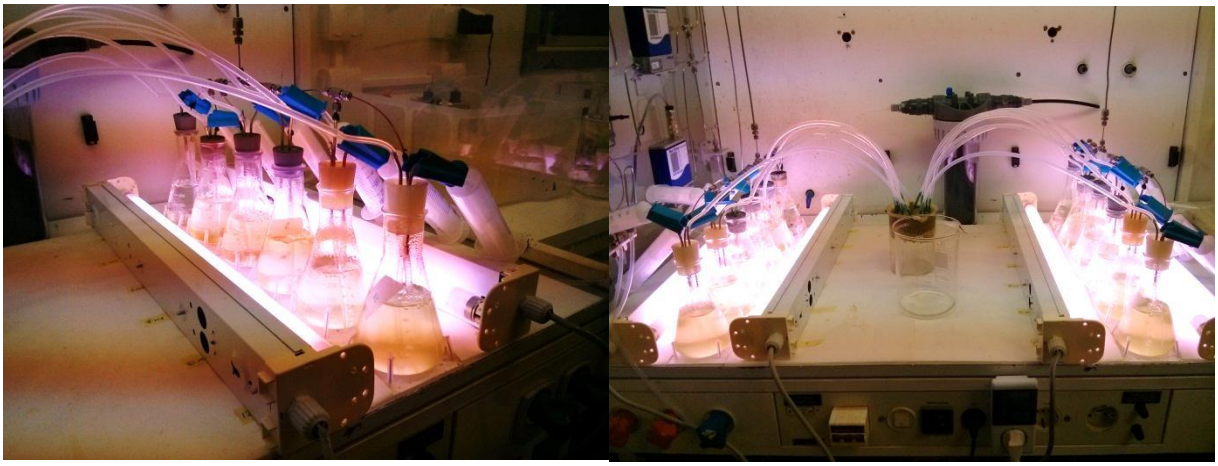


Figure 21: Photos of the growth screening setup. The two rows of cultures are seen between the fluorescent lights. The white tubing seen from each reactor is the gas outflow tubes, which are combined to form a single gas line. The left image shows a close-up of one row of flasks.

3.3.4. Growth screening 1 of isolated local species on HCl-treated biosludge medium

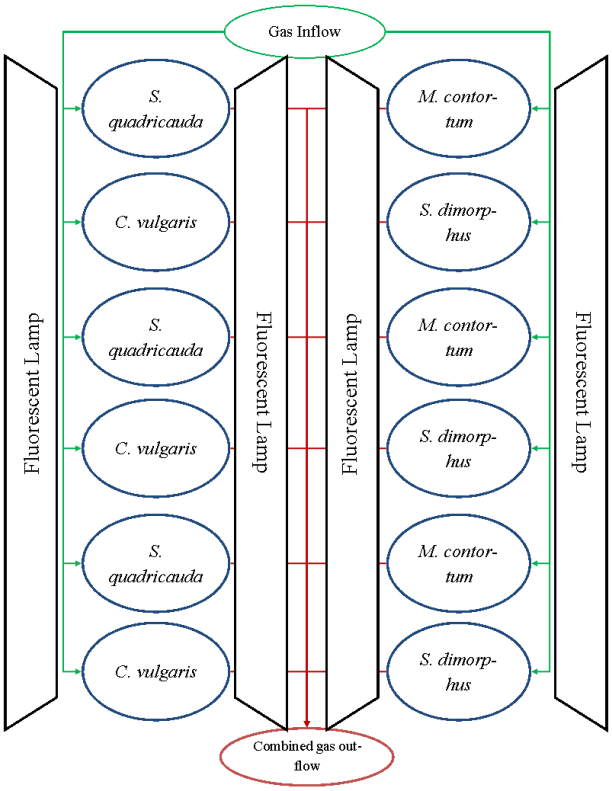


Figure 22: Each selected culture was used in triplicates in this setup. Cultures were spread evenly to minimize any variations between edges and centers of the rows.

Four species were selected for this screening, *S. quadricauda, M. contortum, C. vulgaris* and the local isolate of *S. dimorphus*. The growth of the algae species was investigated in a medium constructed from acid treated biosludge, together with a gas flow of CO_2-enriched compressed air. The initial OD_{750} was 0.05. Pre-cultures were centrifuged at 1100 xg for 5 min, and the supernatant was discarded. The pellets were resuspended in 35 ml of the corresponding medium used for the screening. This was done to remove any nutrients from the 3N-BBM+V culture medium before start. The calculated amount of algal solution was then inoculated in triplicates and gas flow was added as previously shown in Figure 19.

Medium preparation

Two identical batches of growth media were formulated and then mixed to give a single medium. Each batch consisted of 50 g dried biosludge dissolved in one liter 10% HCl for 1 week. Remaining fibers were filtered with Munktell 3 grade filter and the filtrate was collected and pH-adjusted to pH 7 with 5 M NaOH. A large quantity of rapidly sedimenting particles was observed in both formulations. The liquid was decanted and the sediments filtered with Munktell 00A filters. The filtrate from both batches was combined to a single growth medium, used in growth screening 1 of isolated species.

3.3.5. Growth screening 2 of isolated local species on HCl-treated biosludge medium

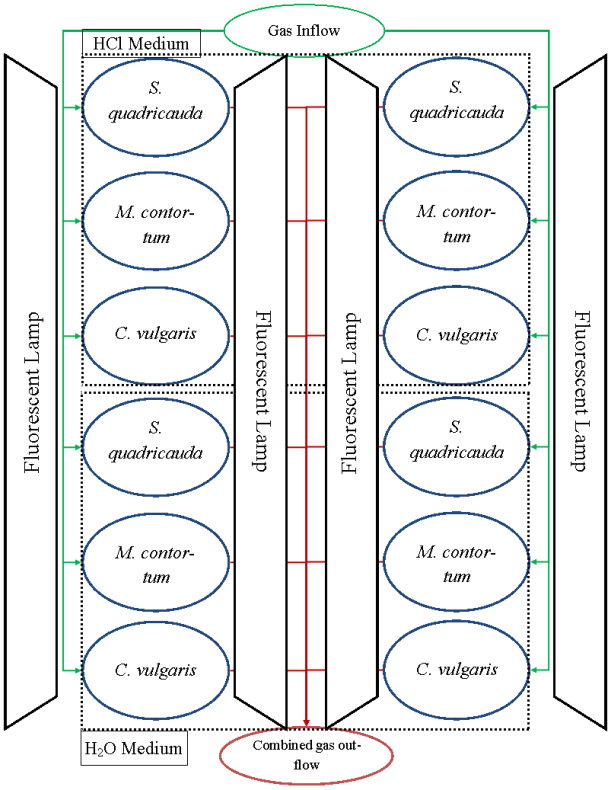


Figure 23: Shown here is the setup used in this screening. Two different media were tested together with 3 separate algae species in duplicates. Each duplicate was placed in each row, with the top three rows containing HCl based medium and the bottom three rows H_2O based medium.

Three local species, *S. quadricauda*, *C. vulgaris* and *M. contortum* were selected for investigating possible medium formulation methods from biosludge. Two methods were investigated; dissolving biosludge in 1 % HCl and heated at 100 °C for 5 days, and dissolving biosludge in deionized water and heated at 100 °C for 5 days. A working volume of 200 ml was used.

Medium formulation

For each medium, 75 g dried biosludge was dissolved under stirring in 500 ml deionized water. The solutions were then diluted to a volume of 1500 ml. The 1 % HCl medium was diluted with 960 ml deionized water and 40 ml concentrated 37 % HCl. The H_2O medium was diluted with 1000 ml deionized water. The acid medium was then partitioned in several sealed 100 ml PTFE chambers and placed in a monitored, ventilated oven kept at 100 °C for 5 days. The neutral medium was poured into a 2l flask, which was sealed and placed in the same oven for the same time period. After 5 days both media were slowly cooled to room temperature and filtered through Munktell 3 grade filters. The filtrates were collected and adjusted to pH 7 with 5 M NaOH.

3.4. Analytical methods

Different tools have been used to evaluate and monitor the different cultures and to follow progress in reactors and culture flasks.

3.4.1. Optical density and pH measurements

Optical density, or synonymously turbidity, (OD) has been used to follow growth. 1.5 ml samples were transferred from reactors to Eppendorf tubes and analyzed in 1 ml cuvettes in a Varian 50 Biospectrophotometer. Blanks were drawn prior to algal inoculation and continually used when measuring OD from all collected samples. The background probably changes during the cultivation, but was assumed to be constant for simplicity. OD was measured at 750 nm with duplicate measurements of each sample, where each measurement was the average of three readings. All reactor vessels were manually shaken prior each sampling to ensure thorough mixing of all cultures.

pH was measured with a SympHony SP80PD pH-meter to assess pH-variations during algae growth. 2 ml samples were drafted from the reactor tanks and transferred to a cuvette for measurements.

3.4.2. Optical microscopy and cell counting

Algae culture samples were evaluated optically using a Dialux 20 microscope and a Visicam 5.0 camera. Images were taken with 400x and 1000x magnification. A Bürker chamber allowed for a method to count cells in different samples. 200 µl cell suspension was added to the chamber and placed in the microscope. For cell counting 15 E-squares were selected at random. All cells within the squares together with those touching the top and left borders were counted, averaged and multiplied with the factor 40 000 to obtain cells/ml. The factor is based on the volume of the E-square, which is 0.025 µl.

3.4.3. Dry weight measurements of produced final biomass concentrations

The dry weight of produced biomass was determined from duplicate 50 ml samples collected from each reactor. The samples were dried in a ventilated 80 °C oven over night. Duplicate 50 ml background samples were also drawn, to be subtracted from the background matrix in all reactors. It was not possible to identify the exact volume of the original medium still present at the end of the cultivation period. A theoretical maximum background weight was therefore subtracted from the produced biomass instead.

3.4.4. CHN-analysis of biosludge

Approximately 3 g humid sludge was dried in an oven at 60 ^{o}C for 12 h. The heat was then increased to 90 ^{o}C for 2 h and finally to 105 ^{o}C for 1 h until the sample was dry enough to analyze. The dry sample was then weighed, covered in aluminum and pelleted for use in a Leco CHN628 analyzer.

3.4.5. Total lipid content and fatty acid profile assay of isolated local algal species

A lipid assay was performed based on the Folch method [65]. Wet biomass samples were collected at the end of the screening of isolated species on 3N-BBM+V and from screening 2 of isolated species on biosludge-based media. The solutions were centrifuged and the supernatant was discarded. Cells were lysed with a 2:1 mixture of chloroform: methanol and phase separated by an addition of a 0.73 % NaCl in H_2O solution. The oil phase containing the lipids were collected and dried overnight. Lipids were then resolved in hexane and the glycerol backbone was extracted by methanolysis. The remaining hexane phase were collected in crimped GC-vials. For a detailed method, see Appendix I.

4. Results

4.1. Growth of *S. dimorphus UTEX 417* on pre-treated biosludge media

The possibilities to use biosludge as the basis of an algal growth medium were assessed with two screenings. Growth of the alga *S. dimorphus* was measured in several different media based on various biosludge treatments, in order to identify any promising methods of increasing nutrient availability. Both screenings contained positive and negative controls, consisting of nutrient rich medium and untreated biosludge. Untreated biosludge was considered a negative control since effective pre-treatments should yield imroved nutrient availability, and thereby better growth.

4.1.1. Growth screening 1 of *S. dimorphus UTEX 417* on biosludge media

Biosludge screening 1 indicated poor algal growth when using biosludge in nets as the primary nutrient source, compared to reactors with 3N-BBM+V and NPK as growth media. The results from screening 1 are shown in Figure 24. For none of the reactors any significant growth were seen, except for the two controls, resulting in OD_{750} values of 0.16 after 200 h. This is equivalent to specific growth rates of 0.01 h^{-1}. The drop in OD_{750} observed at 330 hours in positive control reactor 2 with 3N-BBM+V medium was due to an obstruction in the tubing that fed compressed air into the reactor. The culture then became CO_2-starved inhibiting growth, resulting in a drastic OD decrease. This might be due to observed aggregate formation and flocculation, which can occur in colonies during stress conditions.

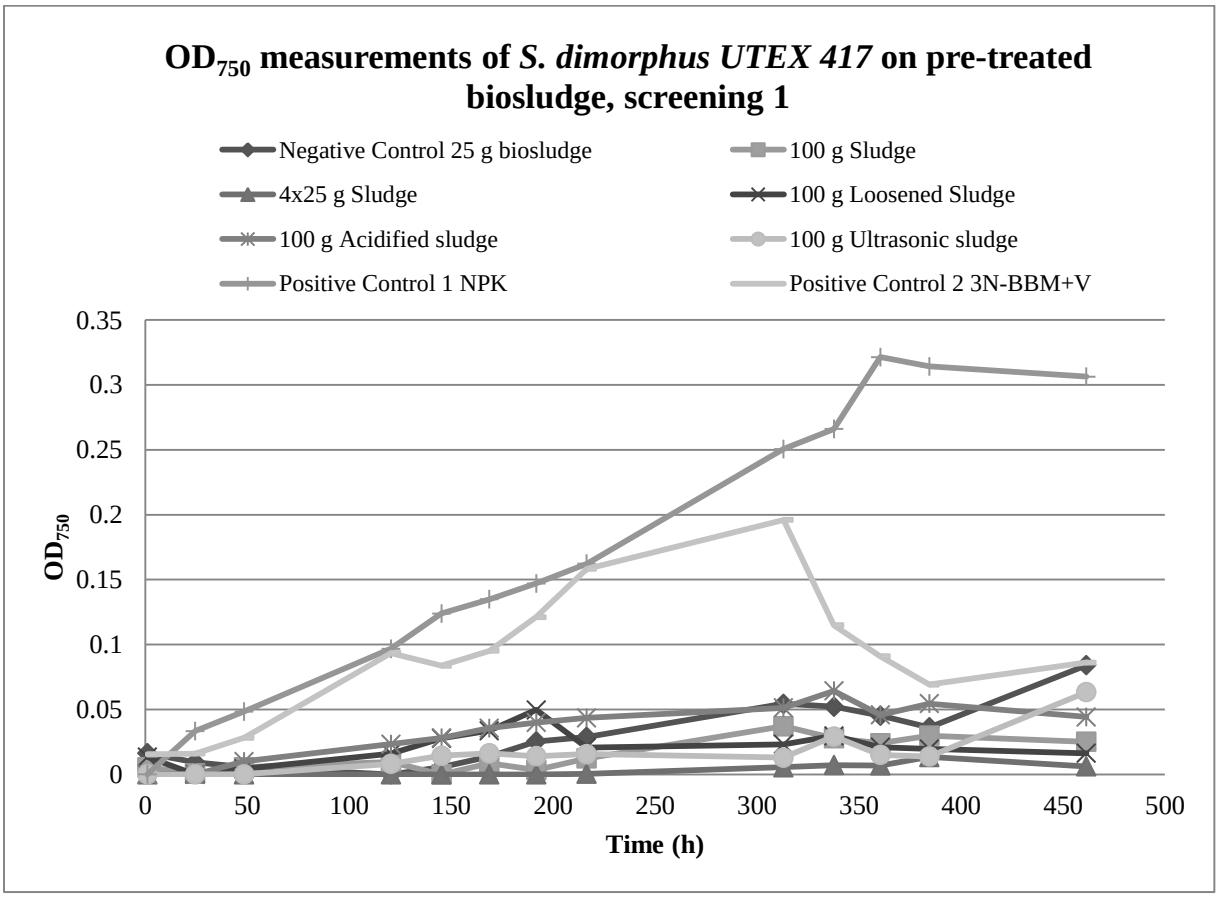


Figure 24: The resulting OD_{750} from the first growth screening of *S. dimorphus* on biosludge-based media. 5 biosludge pre-treatments were evaluated against 2 positive controls of nutrient rich medium and 1 negative control with less biosludge. The drop in OD for positive control 2 was due to a constricted air-flow, resulting in flocculation and aggregation of the alga.

Measured pH-values from biosludge screening 1 are shown in Figure 25. All tanks were adjusted to pH 7 with NaOH before the start of the cultivation and then adjusted again after 24 hours since all tanks showed a significant drop in pH. After that no adjustments were made.

The observed pH-values in all reactor vessels were somewhat stable with most ranging between 7.0 and 8.5. All non-control reactors showed similar pH trends, which was expected since they all showed similar growth behavior. One reactor, the negative control containing less biosludge, showed deviant behavior at the end of cultivation. The pH increased to ~9.5 while all others dropped slightly for unknown reasons.

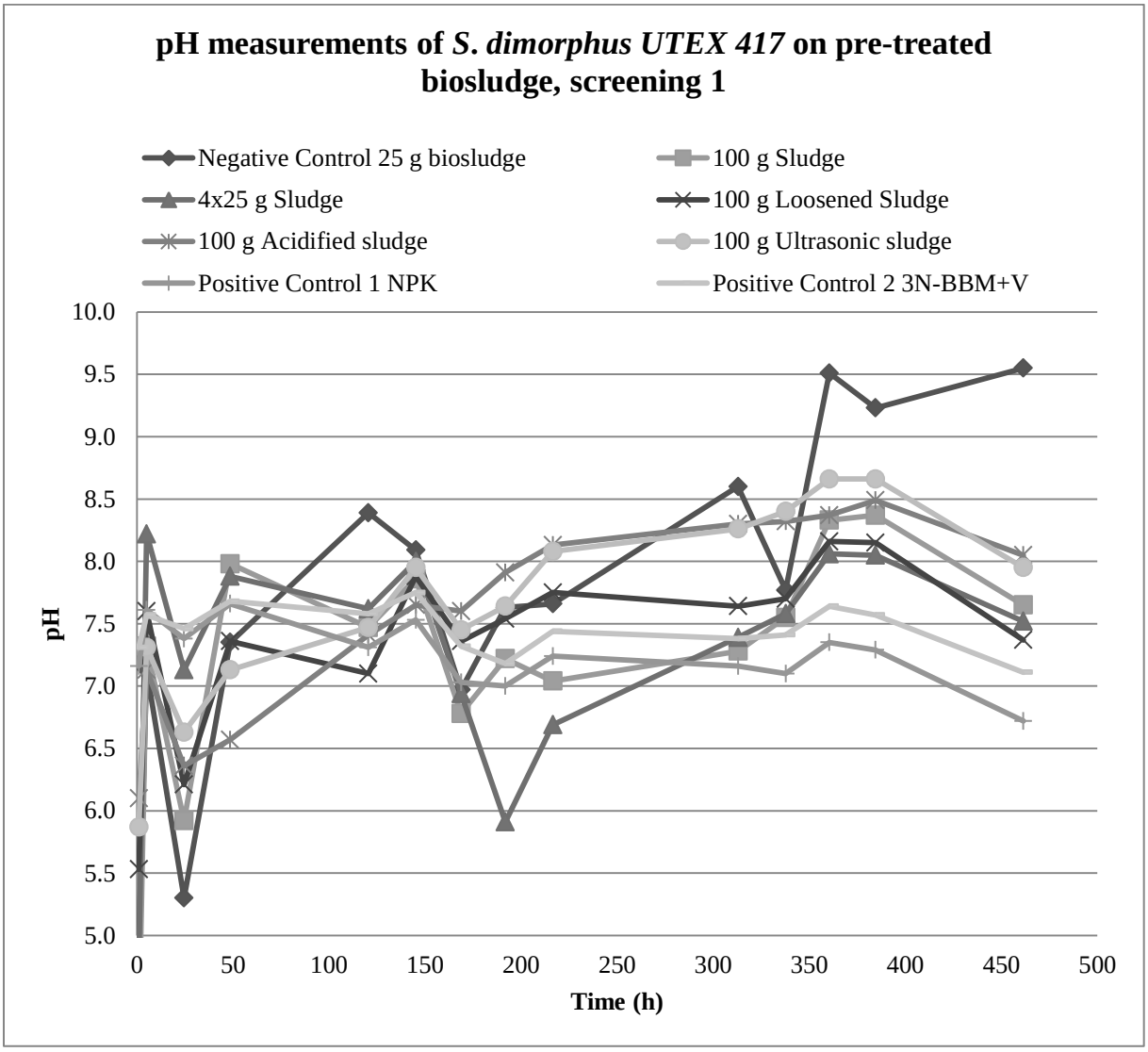


Figure 25: Measured pH-values from growth screening 1 of *S. dimorphus* on biosludge-based media. All reactors were initially adjusted to pH 7 at the cultivation start, and again after 24 h since all showed a significant drop. No more adjustments were made after that. pH was measured in conjunction with OD_{750}-measurements.

4.1.2. Growth of *S. dimorphus UTEX 417* on pre-treated biosludge 2

The second growth screening tested dissolved, pre-treated and filtered biosludge solutions as growth media, illustrated in Figure 26. This screening indicated that both acid and alkaline sludge solutions yielded initially better growth than the controls, but the alkaline pre-treatment caused a heavy aggregate formation resulting in diminishing OD_{750}. Acid sludge filtrate showed promising growth throughout the experiment. However, the OD_{750} values after a growth period of 300 h did not exceed 0.3 for any of the algae cultures. Average algal growth from alkaline sludge filtrate was calculated from only a single reactor, since air supply was accidentally constricted to one of the duplicate reactors and had a great negative effect on the observed growth in that vessel. Growth rates were calculated from logarithmic data and presented in Table 5.

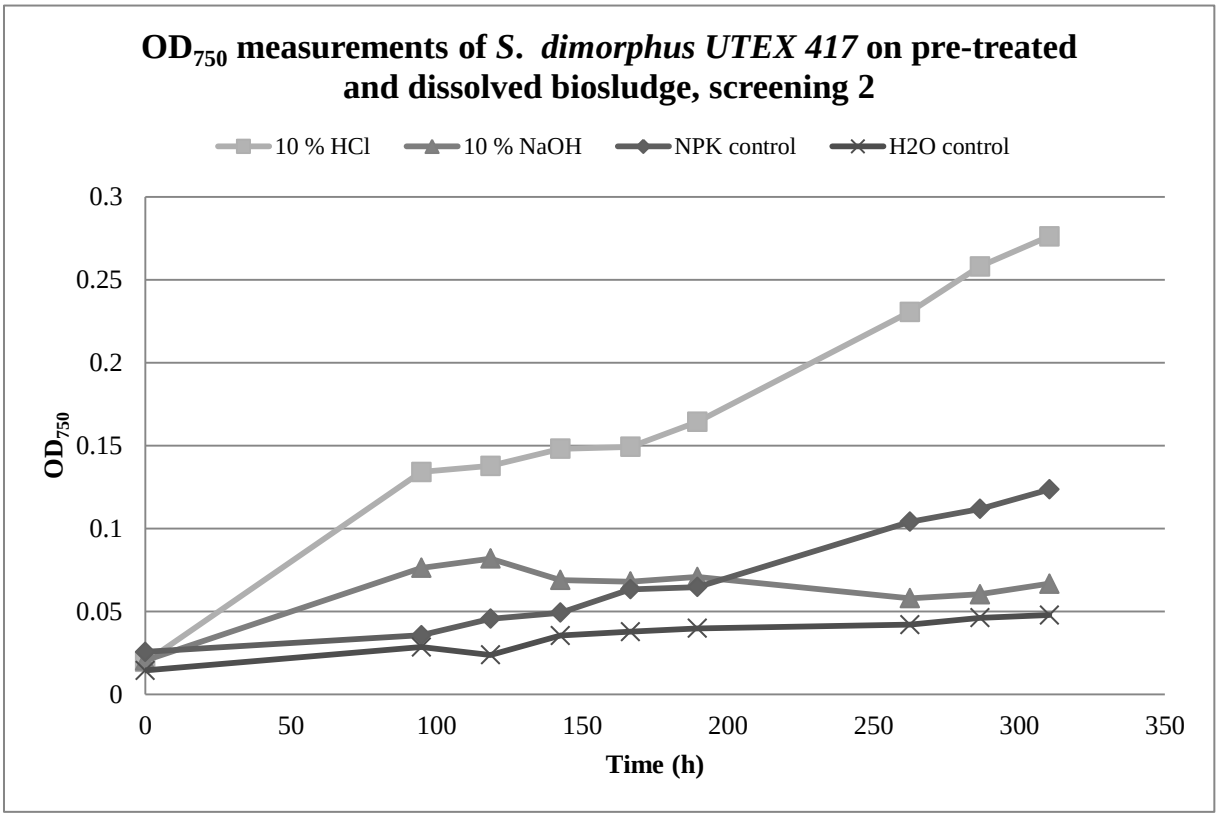


Figure 26: Results from the second biosludge screening. Average growth from alkaline sludge filtrate was calculated from only a single reactor, since air supply was accidentally constricted to the other reactor and had a great negative effect on the OD_{750} measurements in that vessel due to aggregate formation. The fastest growth was observed with acid treated biosludge while untreated sludge yielded the slowest growth.

Growth rates were calculated for two separate phases during the growth, an early phase during the first 100 hours and a late phase between 100 and 300 hours. All reactors but the NPK-controls indicated reduced specific growth rates and largely prolonged generation time for the measured algae compared to reported literature data [41]. Calculated generation times for early phase NPK were much greater than expected. 198.1 h was the longest generation time by far among all cultures; therefore it was also calculated from data for the previous screening. The result from this screening was a generation time of 44.8 hours, similar to both acid and alkaline pre-treatment results. This is probably a more true value, considering the significantly lower generation time at late phase NPK compared to remaining cultures.

Table 5: Specific growth rates and generation times during early and late phases of the second growth screening of *S. dimorphus* on pre-treated biosludge. Acid treated sludge indicated the highest growth rate initially, and NPK resulted in the best growth rate at a late phase. Alkali treatment proved good growth rates initially, but were shown to be very low at a late phase. Biosludge dissolved in water resulted in the lowest growth rates at both phases.

Specific growth rates and generation times of S. dimorphus UTEX 417 cultivated in pre-treated biosludge, screening 2

Phase and pre-treatment	*Specific growth rate (h^{-1})*	*Generation time (h)*
Early phase NPK	0.0035	198
Early phase Water	0.0072	96.8
Early phase Acid	**0.0203**	**34.1**
Early phase Alkali	0.019	36.6
Late phase NPK	0.0058	120
Late phase Water	0.0018	390
Late phase Acid	**0.0043**	**161**
Late phase Alkali	0.0028	245

pH-values from the second screening are shown in Figure 27. A pattern emerged with two visible groupings. NPK and acid treated sludge remained around pH 7 while water and base pre-treated sludge increased to > pH 8.5. *S. dimorphus* are reported to be able to grow within a wide pH-range of 5-10, indicating that the values reported here are of no concern regarding any negative effects on the cultures.

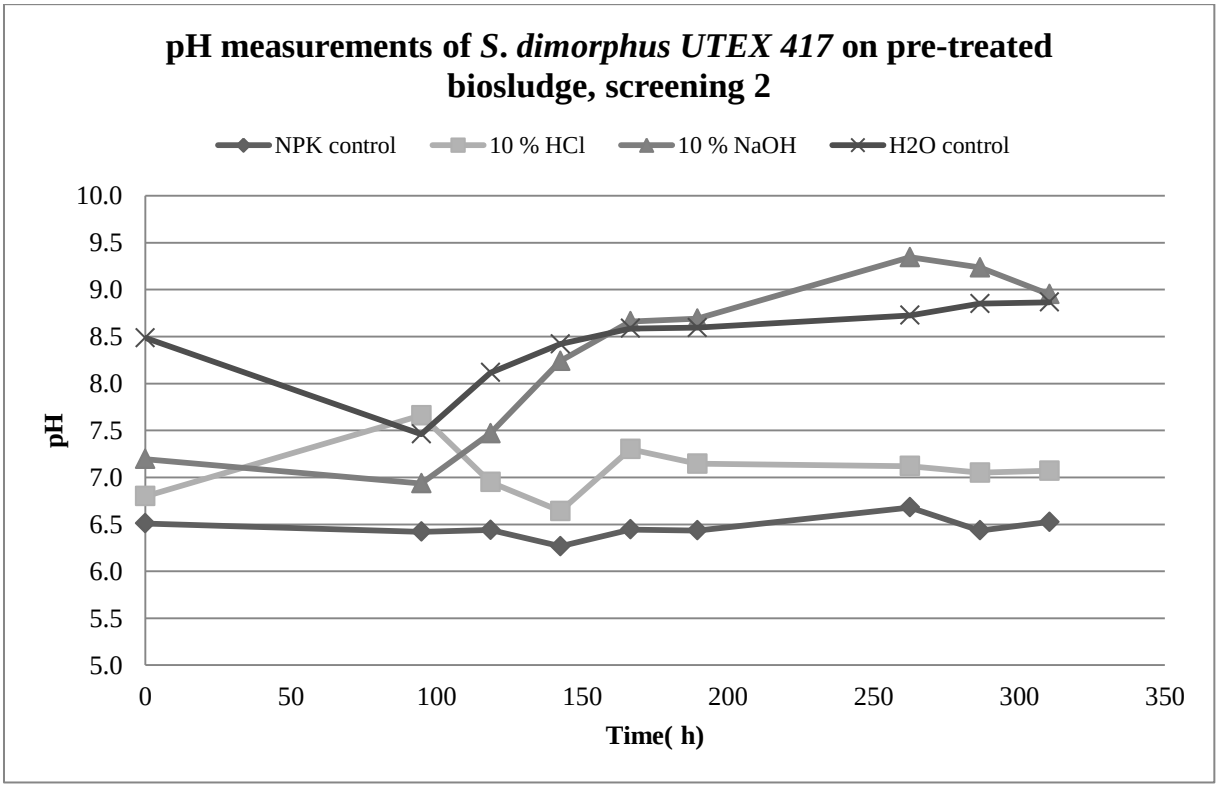


Figure 27: pH values from the second biosludge screening. The algae cultures growing in biosludge medium treated with 10 % NaOH or H_2O indicates rising pH values throughout the cultivation and reaches pH 9 and above at the cultivation end.

Dry weight measurements of the produced algal biomass were done at the end of this screening, see Figure 28. All cultures contained a low biomass density; with no culture reaching 0.4 g/l. The biomass concentration in cultures where the biosludge was dissolved in water was almost nil (0.03 g/l), whereas both acid and alkali treatments produced more biomass (0.38 and 0.24 g/l respectively) than the NPK-control (0.11 g/l).

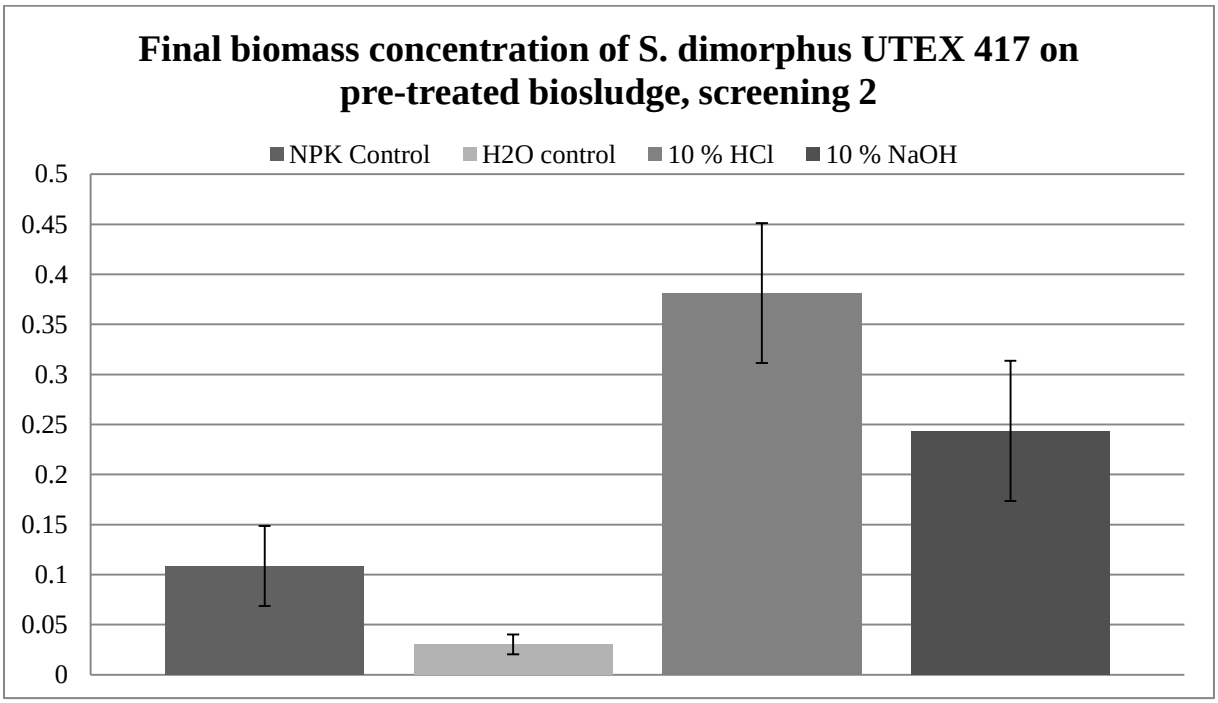


Figure 28: The resulting biomass concentrations for each pre-treatment method are presented in the figure. The highest concentration (0.38 mg/l) was found in cultures cultivated in acid-treated biosludge medium. 0.25 mg/l was observed in cultures cultivated in alkaline-treated biosludge medium. Both concentrations were greater than those observed in reactors cultivated in NPK medium, as well as those cultivated in water-treated biosludge medium.

4.2. Nutrient leaching measurements from HCl-treated biosludge

The effect of reaction time and temperature on nutrient leaching from biosludge treated in 10 % HCl was studied by determining levels of N- and P-containing compounds with Hach-Lange cuvette tests. Measurements were done after 1, 24, 48 and 170 h at 25, 70 and 120 °C. The filtrates from each treatment were observed visually for any differences. 4 filtrate examples are shown in Figure 29, where it is shown that different treatments produced different colorations. The liquid of the mildest treatment (25 °C for 1 h) is pale yellow, and the colors then systematically darken to finally become dark brown, almost black in color.

A darker coloration might be problematic if light inhibiting conditions arise. It was also observed by visual comparisons that the biosludge fibers were degraded to much higher extent for runs with a high temperature and long run-times, resulting in much larger fiber content in the filtrate.

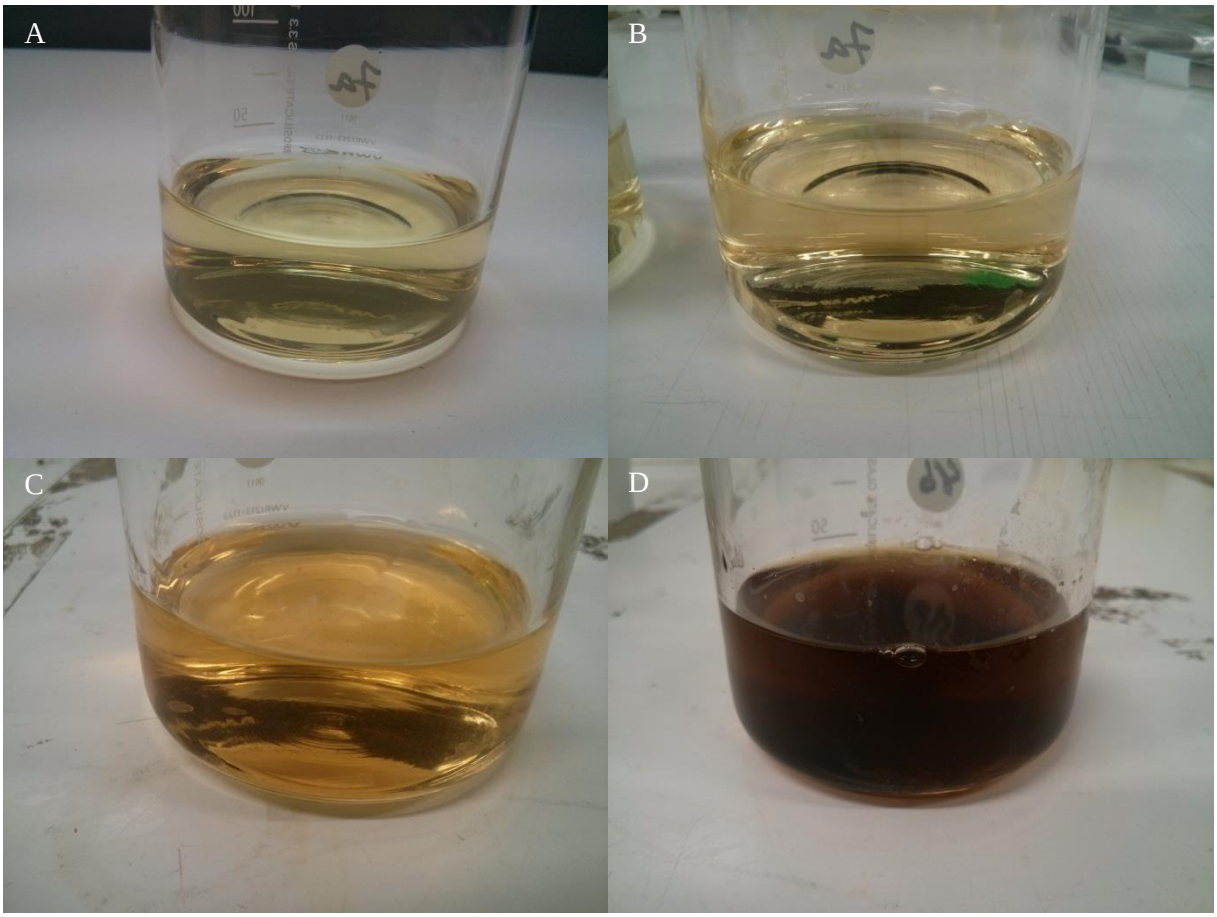


Figure 29: The nutrient leaching screening of pre-treated biosludge yielded visually different filtrates. The test conditions for these 4 images were, from top left to bottom right, 25 °C 1 h, 25 °C 48 h, 70 °C 1 h and 120 °C 24 h. Different colorations were observed for different reaction time and temperature combinations, ranging from pale yellow at the top left (25 °C and 1 h) to dark brown at the bottom right (120 °C and 24 h). It was also observed that for combinations yielding darker filtrates, the filtered fibers were smaller and degraded to a higher extent. The coloration is most likely due to the presence of lignin according to Maria Sandberg at Karlstad University, personal communication.

4.2.1. Measured nutrient concentrations from 10 % HCl treated biosludge

Results of the nutrient leaching from biosludge treated in 10 % HCl compared to reaction time and temperatures are shown in Table 6. Results were grouped together for each nutrient in order to show effects from temperature and time on leached concentrations.

Table 6: Measured concentrations from the different parameter combinations. The parameters for each run are specified to the left with the resulting absorbance for each nutrient in the middle. Standard curves were constructed for each nutrient and used to translate the recorded absorbance to concentrations, which are presented to the right.

Nutrient leaching results from biosludge treated with 10 % HCl

Parameters		*Concentration mg/l*		
Temp (°C)	*Time (h)*	*NH_4^+*	*NO_3^-*	*PO_4^{3-}*
25	1	1.59	5.53	17.2
25	24	2.02	22.3	26.0
25	48	3.49	51.1	31.3
25	170	6.43	64.0	57.0
70	1	2.83	6.95	37.9
70	24	12.6	26.1	58.4
120	1	14.3	9.93	57.1
120	24	19.3	38.3	60.4

NH_4^+ concentration in leachate

Figure 30 presents the resulting NH_4^+ concentrations from biosludge leached in 50 ml 10 % HCl at various reaction times and temperatures. A strongly increased concentration was found when utilizing the highest temperature (120 °C) compared to both 25 °C and 70 °C. The theoretical maximum amount NH_4^+ for this experiment was 18 mg/l based on data provided by NPB, which was reached when using 120 °C for 24 h. Samples measured at 25 °C showed minor differences during the investigated time periods, 1 h resulted in 1.6 mg/l and 170 h resulted in 6.4 mg/l. This indicates that temperature is a more important factor than time when leaching NH_4^+. When comparing concentrations after 1 h at different temperatures, it was found that higher temperatures results in greater concentrations. NH_4^+ concentrations reached 2.8 mg/l in 70 °C at 1 h, and 14.3 mg/l in 120 °C at 1 h. The three highest concentrations were reached with 120 °C 24 h, 120 °C 1 h and 70 °C 24 h.

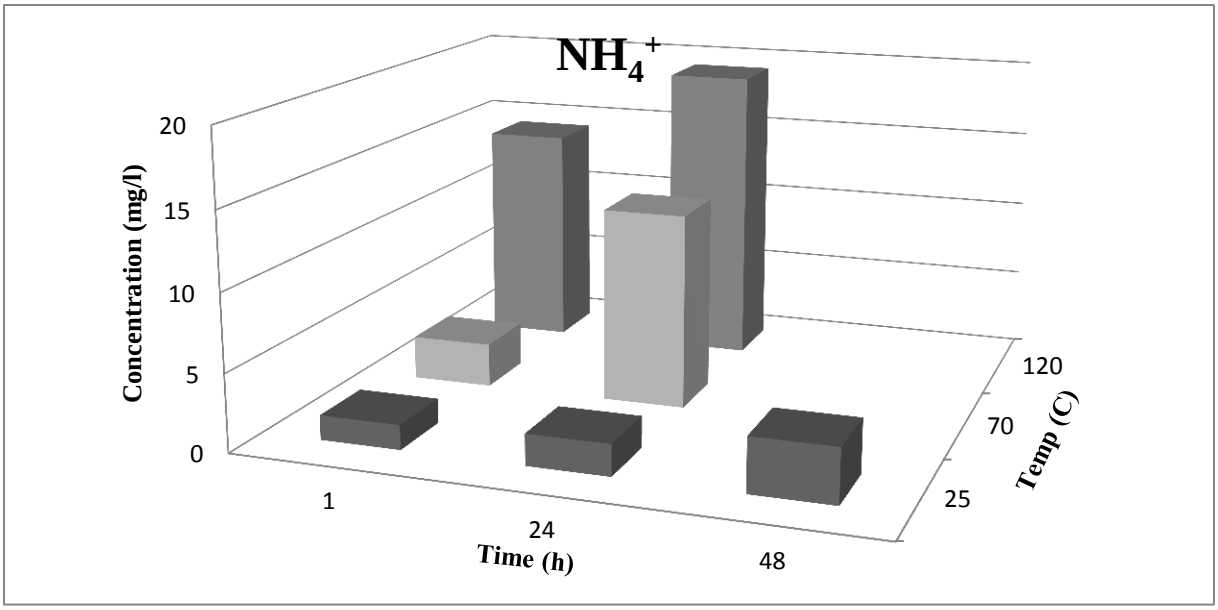


Figure 30: Results of leached NH_4^+ for different times and temperatures. The x-axis shows the different times used (1, 24 and 48 h). Temperatures are at the z-axis (25, 70 and 120 °C) and resulting concentrations are represented by the height of each column. The theoretical maximum concentration of NH_4^+ was reported as 18 mg/l and was reached in three combinations, 70 °C 24 h, 120 °C 1 h and 120 °C 24 h.

NO_3^- concentrations in leachate

Leached NO_3^- concentrations are presented in Figure 31. Compared to NH_4^+, NO_3^- indicates different correlations between time and temperature to yield greater concentrations. Reaction time appears to be the more important factor of the two, as concentration yields were found to be approximately similar at 1 h and 24 h for all temperatures. Concentrations at 1 h were 5.5 – 10 mg/l and at 24 h 22-38 mg/l. The greatest yield was recorded in the combination of 25 °C 170 h with 64 mg/l and the next highest at 25 °C 48 h, which were the two longest reaction time periods in this experiment. It was still found that higher temperatures yielded greater nutrient concentrations, indicating a positive correlation as expected. Yields were found to increase 4-fold between 1 h and 24 h, and as much as 10-fold between 1 h and 48 h. No theoretical maximum could be specified for this experiment due to lack of data from NPB.

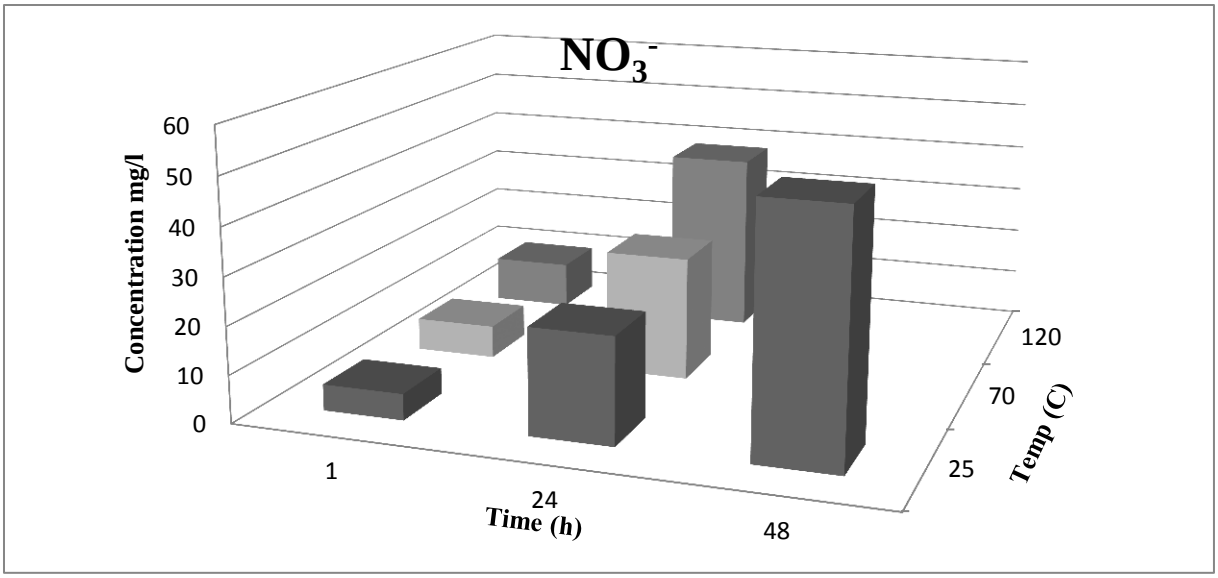


Figure 31: Resulting NO_3^- concentrations with different times and temperatures. The x-axis shows the different times used (1, 24 and 48 h). Temperatures are at the z-axis (25, 70 and 120 °C) and resulting concentrations are represented by the height of each column. No data for theoretical maximum concentrations of NO_3^- was reported.

PO_4^{3-} concentrations in leachate

Figure 32 present the resulting PO_4^{3-} concentrations. The theoretical maximum was 60 mg PO_4^{3-} based on data from NPB, which is reached in 3 separate runs; 70 °C 24 h, 120 °C 1 h, and 120 °C 24 h. Concentration of leached PO_4^{3-} were quite high in all combinations, with the lowest concentration being 17 mg/l at 25 °C 1 h. PO_4^{3-} concentration was almost doubled between 25 °C 1 h to 25 °C 48 h, from 17 to 31 mg/l. PO_4^{3-} does not appear to be a limiting factor for algal growth, as nitrogen is needed to a greater extent according to the classic Redfield ratio of 106 C : 16 N : 1 P [71].

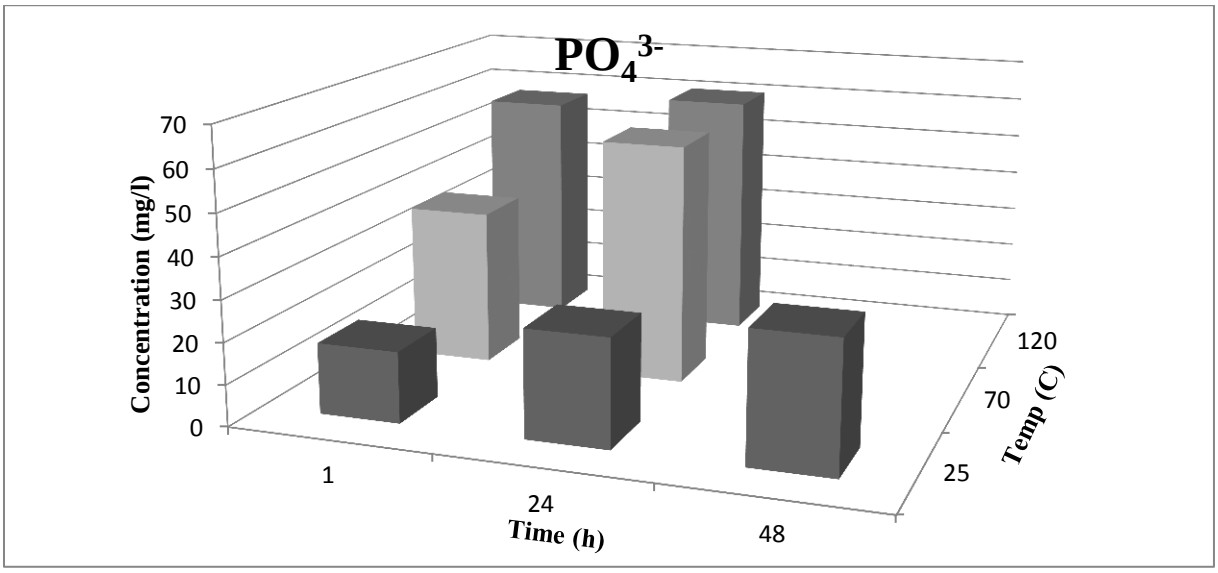


Figure 32: Resulting PO_4^{3-} concentrations for the different runs. The x-axis shows the different times used (1, 24 and 48 h). Temperatures are at the z-axis (25, 70 and 120 °C) and resulting concentrations are represented by the height of each column. The theoretical maximum concentration of PO_4^{3-} was reported as 60 mg/l and was reached in three combinations, 70 °C 24 h, 120 °C 1 h and 120 °C 24 h.

Nutrient concentrations after one week treatment of biosludge

The effects of longer reaction times were investigated by an experiment running for one week at 25 °C. The results from this treatment can be found in Figure 33, where the concentrations are compared to the previous top notations from each molecule. After one week at 25 °C, concentrations for the three nutrients reached the following: NH_4^+ 6.4 mg/l, NO_3^- 64 mg/l and PO_4^{3-} 57 mg/l. The previous top notations for both NH_4^+ and PO_4^{3-} were recorded in the same run, at 120 °C and 24 h where the leached concentrations reached 19 and 60 mg/l respectively. The top concentration for NO_3^-, 51 mg/l, was recorded at 25 °C and 48 h. NH_4^+ is the only one of the nutrients showing a largely lower concentration after one week, compared to the high temperature run. Results in Figure 30 suggested that temperature was of more importance than reaction time, which is further corroborated here. NO_3^- shows a largely increased concentration compared to the previous top notation, also in line with results presented in Figure 31. Here it was considered that the reaction time was more important than the reaction temperature. The PO_4^{3-} concentration reached the theoretical max limit once again, and was therefore similar to the previous top notation.

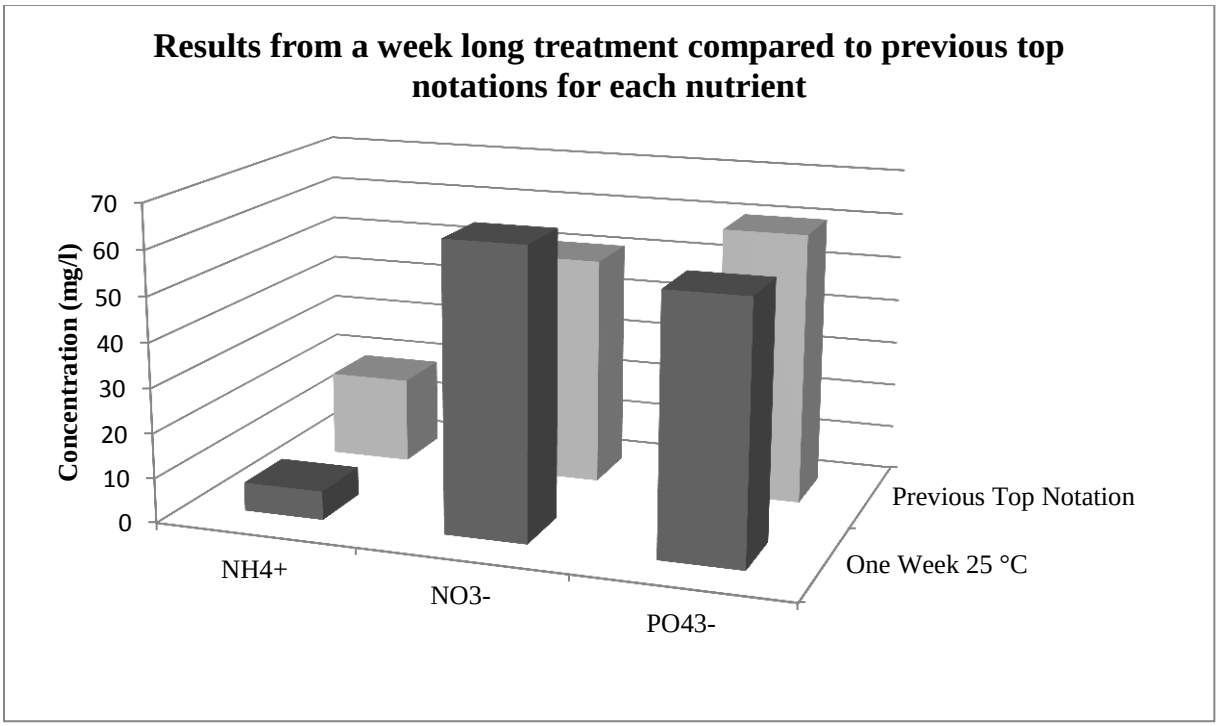


Figure 33: The leaching results from biosludge placed in an acid environment for a week shows that a majority of the theoretically available PO_4^{3-} is released in the filtrate, together with roughly 65 mg/l NO_3^-. NH_4^+ does not show major differences between a 48 hour and 1 week treatments.

4.3. Isolation of local algae species from NPB

Of the 18 colonies transferred from agar plates to 50 ml tissue culture flasks 16 were found to be pure cultures containing a single algal morphology. Photos of the isolated species are shown in Figure 34 to Figure 38. A total of seven different morphologies were found among the 18 flasks and were screened for suitable growth rates. Morphologies were identified via the identification guide by John *et al* [39]. Many more species were found in the samples, but were not isolated.

Two filamentous species were identified as *Phormidium sp* and *Oscillatoria sp*. shown in Figure 34. Both species showed distinctly unique morphology due to their filamentous growth and slight difference in color, with *Phormidium sp* being a slightly darker green, and the formation of a mucus layer from *Oscillatoria sp*.

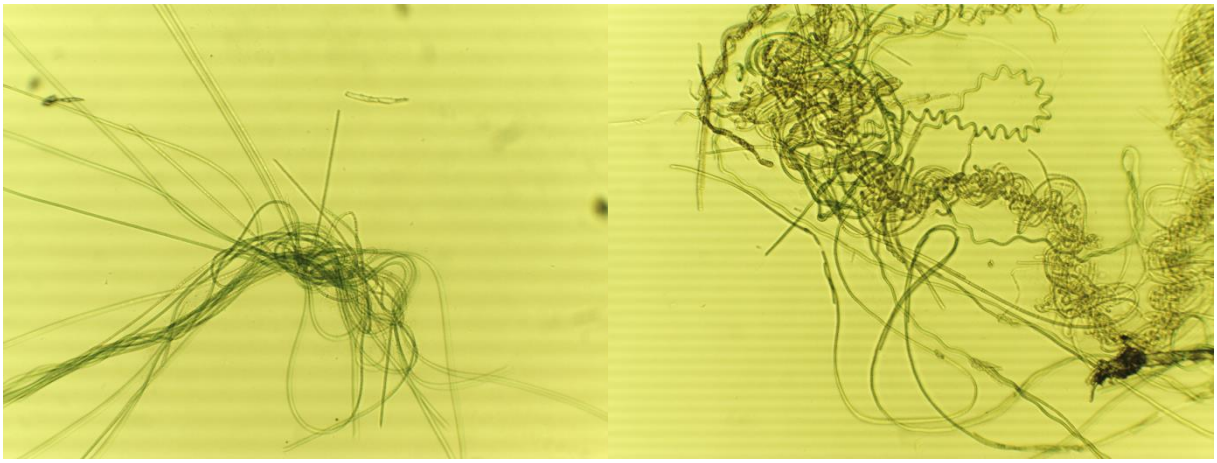


Figure 34: The two isolated filamentous algae species *Phormidium sp*. (left) and *Oscillatoria sp*. (right)

Two different species of *Scenedesmus* were found among the isolated cultures, shown in Figure 35, which were *Scenedesmus dimorphus* and *Scenedesmus quadricauda*. The *S. dimorphus* strain might be the previously inoculated strain used for algal cultivation *in situ* at NPB; a genetic strain determination would be required and has not been performed in this project.

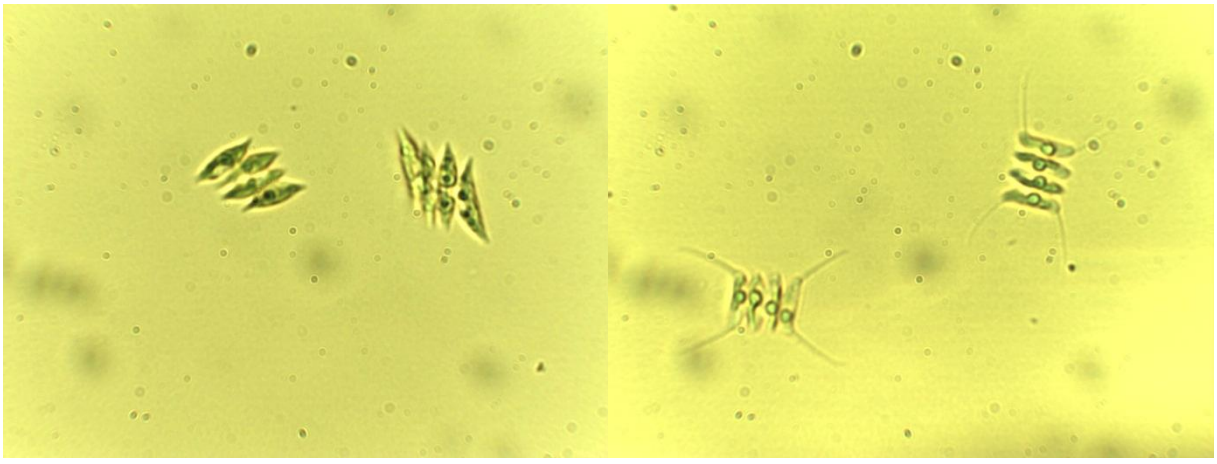


Figure 35: The two isolated *Scenedesmus* species *S. dimorphus* (left) and *S. quadricauda* (right).

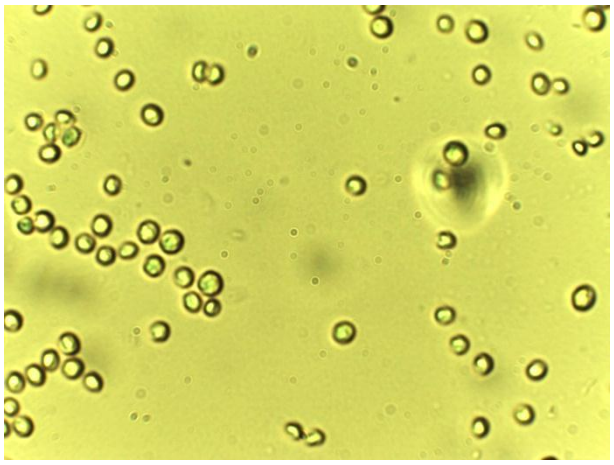


Figure 36: An isolated strain of *Chlorella vulgaris*.

A strain of *Chlorella vulgaris* was isolated and is shown in Figure 36. This alga was found in several growth flasks, indicating a great abundance of this species at NPB.

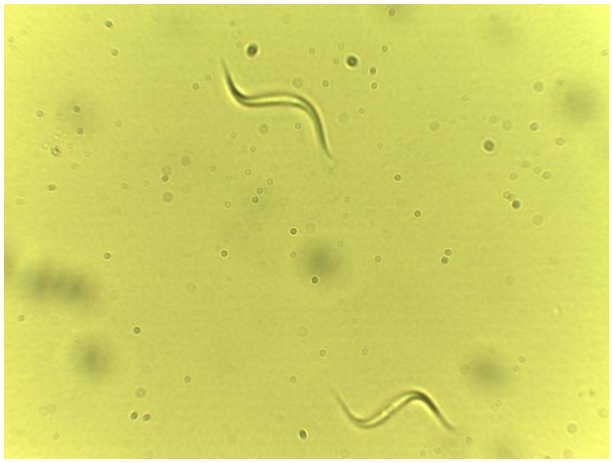


Figure 37: *Monoraphidium contortum*. These spindle like cells were found in several culture flasks and were prone to contaminate other cultures.

Almost half of the isolated cultures consisted of a *Monoraphidium* species, *Monoraphidium contortum* shown in Figure 37. This alga was observed in 7 of 18 flasks, mostly as pure monocultures, but also as a part of a co-culture with other algae species. It was not possible to determine if it was one or several different strains. *M. contortum* showed promising growth in 3N-BBM+V medium but not in growth media based on biosludge.

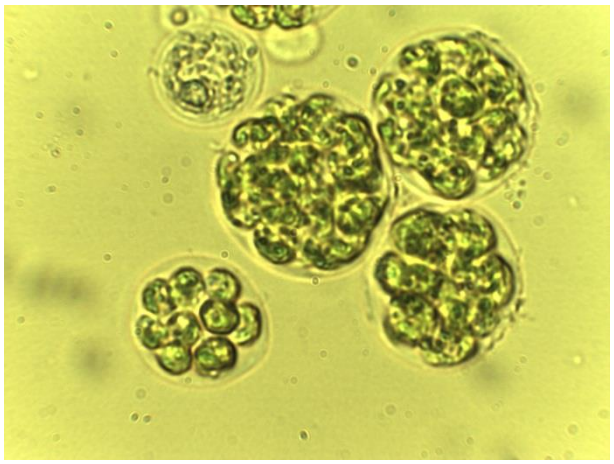


Figure 38: *Coenococcus sp*. This alga grows as a coenobia of cells and was of great interest due to its unique features. No data could be obtained on this species as it died after 1 week in the tissue culture flasks.

The alga shown in Figure 38 was a coenobia of several cells, and was deemed as a species *Coenococcus*. This was the only coenobia found among the isolated cultures, but this alga did not survive the initial cultivation in growth flasks. No cells were found after 1 week of culturing. Subsequently, this species was never part of any of the following experiments.

4.4. Growth rate screenings of isolated algae species

Three growth screenings were perfomed with different growth media and several selected algae species. *S. dimorphus, S. quadricauda, C. vulgaris and M. contortum* locally isolated at NPB, together with the previously inoculated strain of *S. dimorphus* used in the algae experiment at NPB. The first screening was performed with 3N-BBM+V medium as nutrient source, the following two with biosludge-based media pretreated in different ways. A combination of heat and dilute HCl was used to increase leaching of NO_3^-, NH_4^+ and PO_4^{3-}.

4.4.1. Growth rate screening of isolated species on 3N-BBM+V medium

Isolated cultures were stored in 3N-BBM+V medium in tissue growth flasks. Pure cultures with *Phormidium sp, Oscillatoria sp, S. quadricauda, S. dimorphus, M. contortum* and *C. vulgaris* and the strain used for inoculation at NPB, *S. dimorphus UTEX 417*, were selected for a growth rate screening with CO_2 content up to 15 %. The two filamentous species were given a single reactor each, while remaining cultures were evaluated in duplicates.

Resulting OD_{750} values are presented in Figure 39. All cultures except the filamentous species showed promising fast growth with growth rates exceeding 0.05 h^{-1}. Both reactor vessels of *S. dimorphus* 1 were found to be highly contaminated by *C. vulgaris* and should be regarded as a co-culture of the two species. Final OD_{750} values differed greatly between the cultures with the co-culture in *S. dimorphus* almost reaching OD 14 after 240 h of growth.

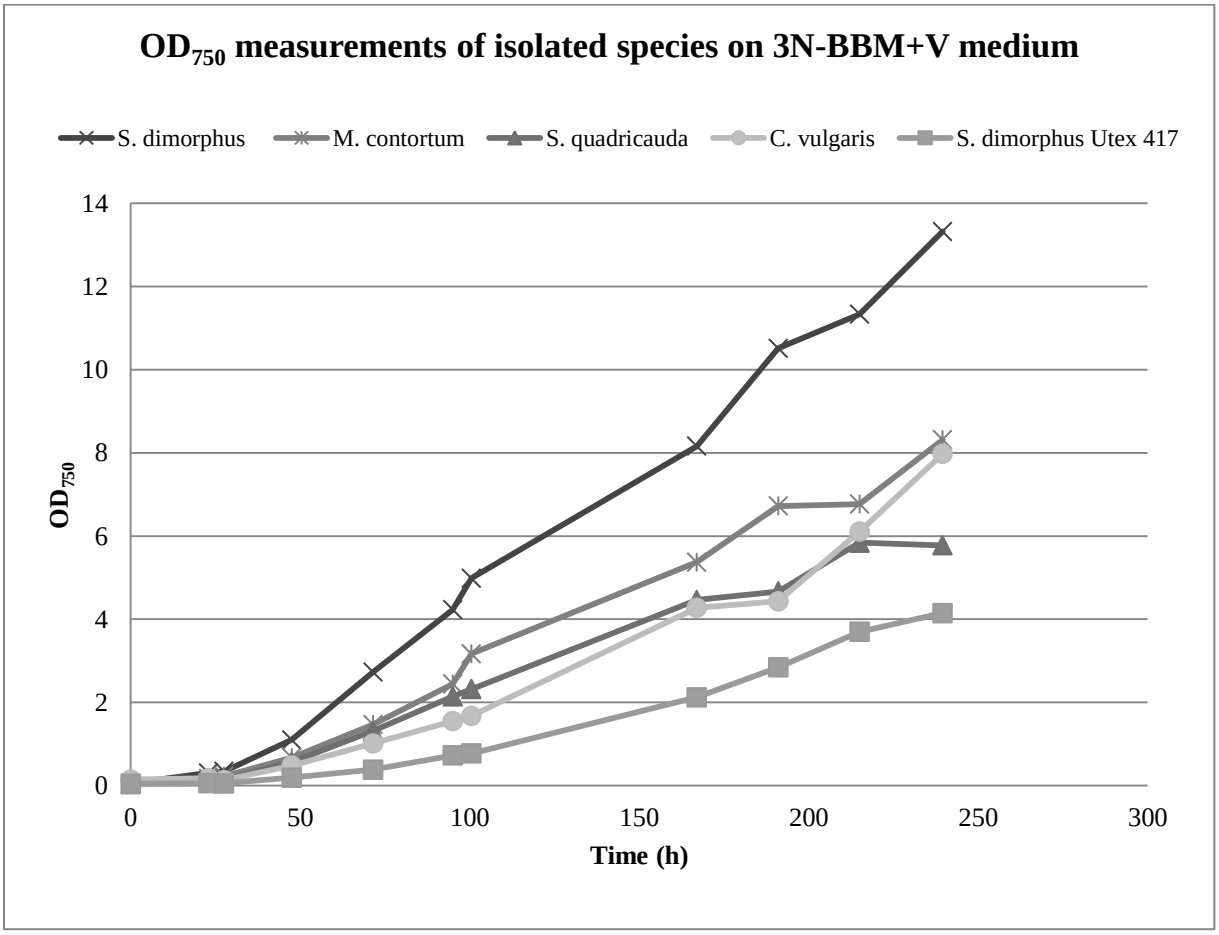


Figure 39: Complete growth results from the growth screening of isolated species in 3N-BBM+V medium with gradually increasing CO_2 content up to 15 %. *S. dimorphus* 1 represents the isolated strain and *S. dimorphus* UTEX 417 represents the previously inoculated strain at NPB.

All cultures grew quite rapidly, visualized in Figure 40 and Figure 41. The color difference was very obvious between 0 and 24 h, as well as between 24 and 48 h. No cultures were found to fully enter the stationary phase although growth was severely diminished at the end of cultivation after 240 h.

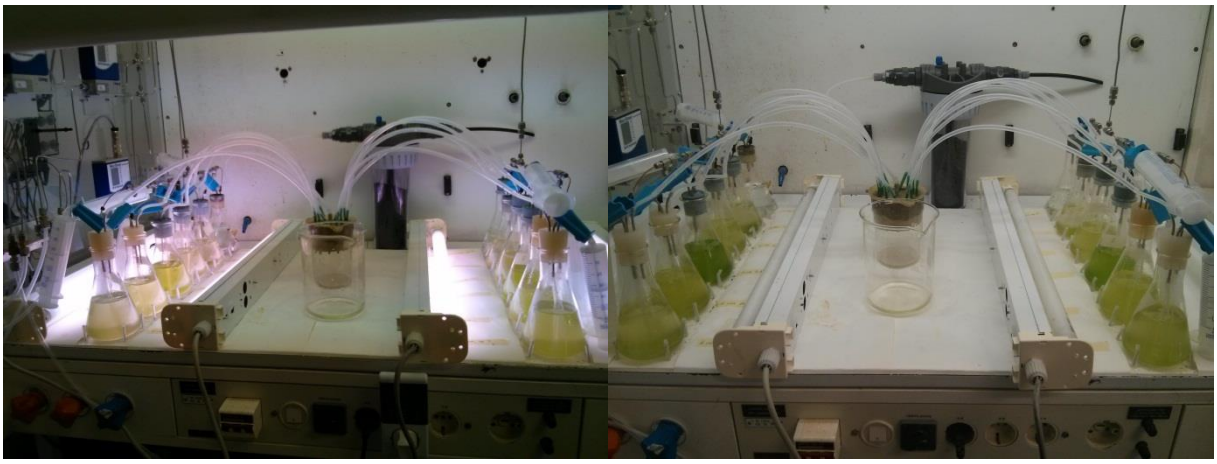


Figure 40: Twelve batch cultures from the flue gas system at two times, 24 h and 48 h, from start are illustrated. The difference between 24 hour growth and 48 hour growth is shown here, 24 hour being the left image. One culture, *S. dimorphus* 1 in the middle, is clearly darker than all others after 48 h growth.

Aggregates large enough to be visible by eye were formed when cultures reached OD values over 5 and manual extra agitation was required to keep cells from forming sediment. The vessel containing the filamentous species *Phormidium sp*, seen in the top right reactor in Figure 41 became contaminated of *M. contortum* after 72 h. This can be seen in the different colors in the vessel between the left and right image.

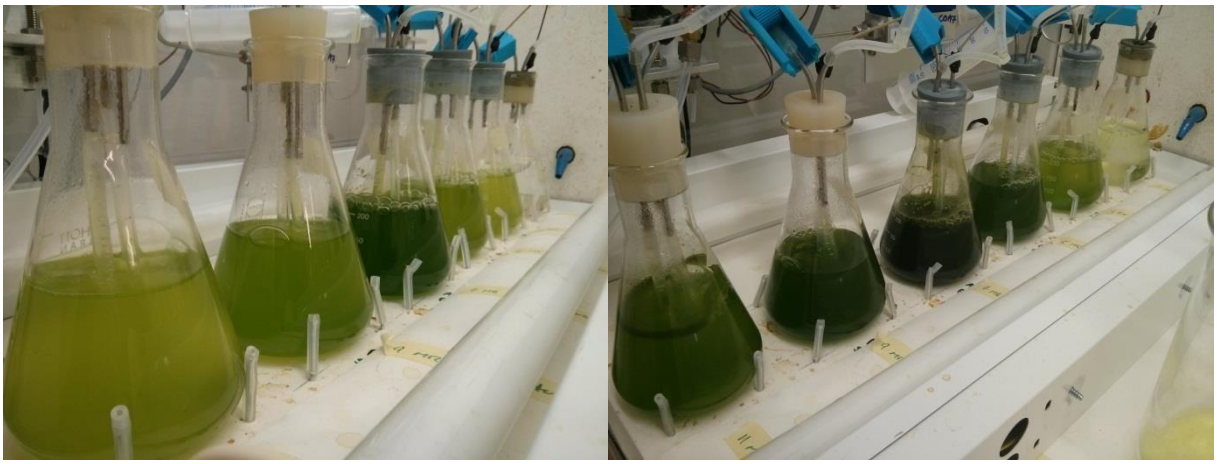


Figure 41: The left image is taken 72 hour after growth initiation and differences in growth are still clearly visible between the cultures. The right image was taken after 96 hours, and the cultures start to get real dark green. It is not thought that the dark color interfered with cell growth, due to the small size and volume of the reactor. Although manual agitation was required to hinder sediments, gas bubbling were considered sufficient in mixing the culture to ensure satisfactory illumination.

The filamentous species, *Oscillatoria sp*, indicated growth initially with clearly visible filaments, but died when CO_2 influx levels were increased to 15%, see Figure 42. The left image is taken 2 h before the increase of CO_2 from 7.5 % to 15 %, and the right is taken 22 h after. All filament fragments had lost color and were broken up in pieces.

Figure 42: *Oscillatoria sp* did not cope well with an environment consisting of 15 % CO_2, and died shortly after the high CO_2-level was initiated. The alga appeared to grow well in 7.5 % CO_2 based on visual observations. The images are taken 24 h apart, the left 2 h before CO_2 levels were increased to 15 % and the right is taken 22 h after.

Specific growth rates, μ (h^{-1}), and generation times, g (h) were calculated from logarithmic data and are presented in Table 7. Specific growth rate and generation time calculations were performed for two separate stages, early phase between 27 and 48 h, and late phase between 100 and 240 h. All tested species resulted in similar growth rates around 0.05-0.06 h^{-1}.

Table 7: Growth rates and generation times for 5 species from the growth rate screening with elevated CO_2 levels of 15 %. Growth rates were calculated at both an early and late stage during growth. Two cultures, *S. dimorphus 2* and *C. vulgaris*, displayed a lag phase before growth was observed. These cultures show a higher growth rate in the late phase than the other three, possibly indicating that these might contain higher nutrient levels than the other cultures and have not yet fully entered stationary phase.

Growth Rates of isolated species on 3N-BBM+V and high CO2 content

		S. quadricauda	*S. dimorphus + C. vulgaris*	*M. contortum*	*S. dimorphus UTEX 417*	*C. vulgaris*
Early phase	μ (h^{-1})	0.057	0.064	0.054	0.062	0.065
	g (h)	12.2	10.8	12.8	11.2	10.6
	Lag-time (h)	0	0	0	27	27
Late phase	μ (h^{-1})	0.007	0.007	0.007	0.012	0.007
	g (h)	106	98.1	100	57.4	106

When the cultivation were. the co-culture of *C. vulgaris* and *S. dimorphus* resulted in the highest final OD_{750} at 13.3, shown in Figure 39. *M. contortum* and the *C. vulgaris* monoculture ended slightly lower at 8.3 and 7.9. *S. quadricauda* and the *S. dimorphus* monoculture were the lowest with 5.8 and 4.1. Late phase generation times for both *S. dimorphus* Utex 417 and *C. vulgaris* suggests that neither yet had entered the stationary phase.

All reactors were terminated simultaneously for dry weight measurements, the results can be viewed in Table 8. The co-culture of *C. vulgaris* and *S. dimorphus* produced twice the amount of biomass, 2.3 g/l, as either the pure *S. dimorphus UTEX 417* culture, 1.1 g/l, or the pure *C. vulgaris* culture, 1.0 g/l respectively. These two species were the least productive of all, while pure colonies of *S. quadricauda* and *M. contortum* showed higher biomass concentrations, but still less than the co-culture at 1.9 and 1.6 mg/l, respectively.

Table 8: Final dry weights of cultures after 240 h growth. The co-culture in *S. dimorphus* 1 produced 2.3 g/l biomass, highest of all cultures. *S. quadricauda* produced most biomass of the pure colonies, while *C. vulgaris* produced 1.0 g/l biomass.

Final dry weight of cultures grown in 3N-BBM+V with 15 % CO_2

Culture	*Dry weight g/l*
S. dimorphus + C. vulgaris	2.3
S. dimorphus UTEX 417	1.1
S. quadricauda	1.9
M. contortum	1.6
C. vulgaris	1.0

4.4.2. Growth rate screening of isolated species on biosludge medium, screening 1

The growth medium for this experiment was formulated from dried biosludge with the nutrition content specified in Table 9. Nutrition data was measured with Hach-Lange cuvette tests before growth start. The conductivity was measured to give an indication of the salinity in the mixture. According to Chemiasoft [72] the following relation exists:

$$87800\ \mu S/cm\ at\ 22\ °C = 6.6\ \%\ Salinity$$

This is extreme conditions for freshwater algae to survive, and a refined method was used in the follow up screening to minimize salinity while not reducing nitrogen and phosphorous levels.

Table 9: Nutrient content of the formulated medium from biosludge. This medium contains about half as much available N compared to 3N-BBM+V medium.

Initial properties and nutrient concentrations for the used biosludge-based growth medium

Nutrients	*NO_3^- mg/l*	45
	NH_4^+ mg/l	16
	PO_4^{3-} mg/l	74
Conductivity µS/cm		87800
Salinity %		6.6

The investigated species were *S. quadricauda*, *C. vulgaris*, *M. contortum* and *S. dimorphus*. The two filamentous species were rejected due to poor growth in previous tests. All 4 cultures were grown in triplicates and the average OD_{750} of each culture was calculated. OD_{750} curves presented in Figure 43 confirmed no existing algae growth in any culture. Instead, all cultures showed heavy bacterial contamination and the experiment was cancelled after just 48 h.

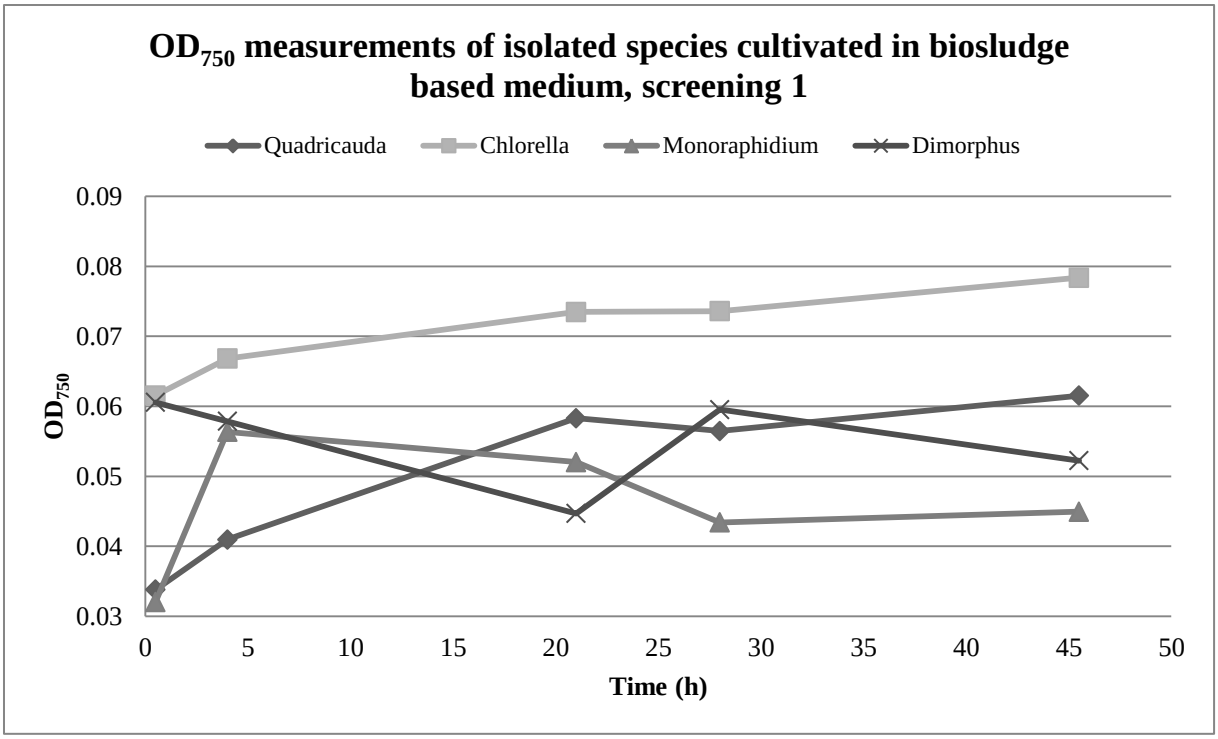


Figure 43: Resulting OD for OD_{750} measurements of isolated species cultivated in biosludge-based medium. After 48 h no growth was observed while all reactors showed major bacterial growth. The screening was cancelled prematurely based on these observations.

4.4.3. Growth rate screening of isolated species on biosludge medium, screening 2

This screening evaluated possible growth of *C. vulgaris*, *M. contortum* and *S. quadricauda* in two different media formulated from dried biosludge. The two growth media used were based on biosludge dissolved in either a 1 % HCl solution or in H_2O. The media where then kept at 100 °C for 5 days, after which they were filtered.

Growth of the three species was followed via $OD_{750,}$ see Figure 44. No growth was observed for any culture in HCl-treated medium. Two of the three species indicated growth in H_2O-treated medium, namely *S. quadricauda* and *C. vulgaris*. *M. contortum* showed zero growth in both media. Final OD_{750} values were similar for the two species, both around OD_{750} 1.4. Initial growth rates were also similar between the two, but *C. vulgaris* exhibited a longer acclimatization period when high CO_2 levels were reached. Gas influx levels of 15 % were used after 75 h, around the same time point were differences in OD can be noticed.

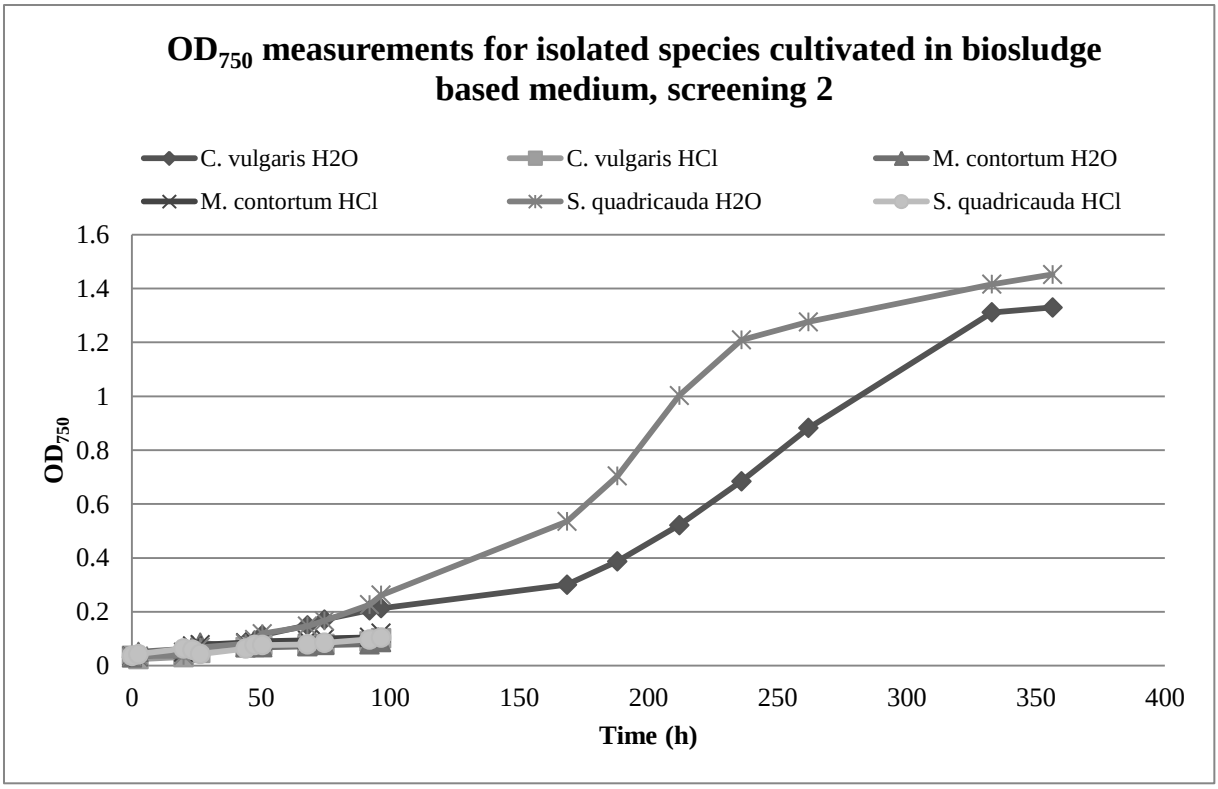


Figure 44: The three species selected for this screening, *C. vulgaris*, *M. contortum* and *S. quadricauda* were grown in two different media fabricated from dried biosludge. The resulting growth graph is presented here. Two of the six cultures showed significant growth, both in medium based on sludge treated in water. These cultures were measured daily for two weeks until a stationary phase were detected for both cultures.

Both media used for this screening were quite heavily discolored, with a strong yellow/brown color. This was observed for both H_2O and HCl-treated media, but more so with the acid. Algal growth was easily observed as the cultures first turned a darker brown, which then shifted to green and dark green. Some images from this experiment can be seen in Figure 45 and Figure 46.

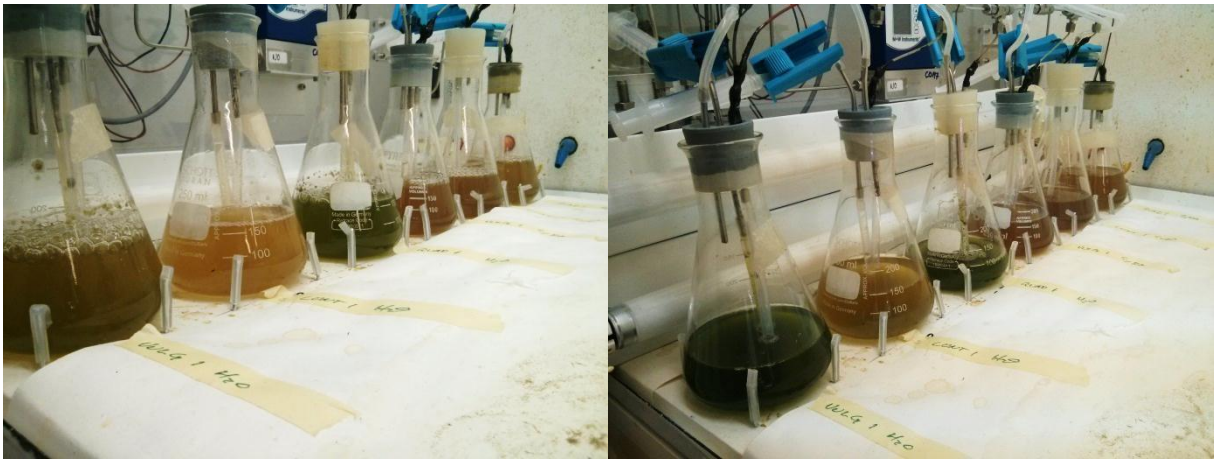


Figure 45: Six of the twelve flasks shown together for comparative purposes. Growth was only observed in two cultures, *S. quadricauda* (middle culture) and *C. vulgaris* (left culture) both in H_2O-medium. The left image is taken after 5 days growth, the right after 2 weeks.

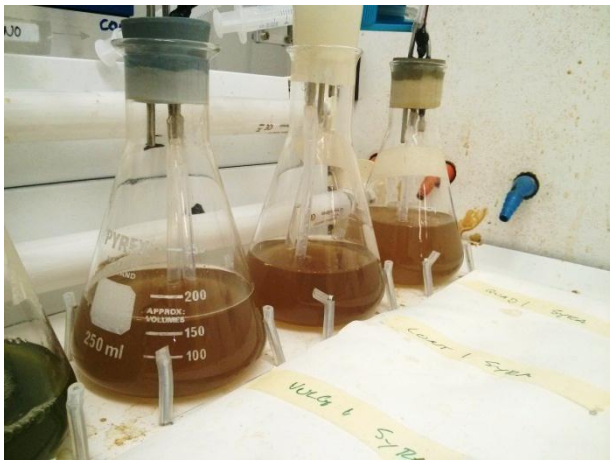


Figure 46: No algal growth was observed in any culture with 1 % HCl based biosludge medium during the experiment. This image was taken after 2 weeks.

Although no algal growth was observed in any HCl-treated biosludge medium, no bacterial growth was found either. This indicates that the treatment sterilizes the biosludge, as opposed to the experiment performed in section 4.4.2.

Except the period of low growth between 100-150 h *C. vulgaris* showed slightly faster growth rates overall, see Table 10. Growth rates were slower and generation times longer than those observed for algal species grown in 3N-BBM+V medium, see Table 7.

Table 10: Growth rates and generation times for two algae species, *S. quadricauda* and *C. vulgaris* in H_2O-based medium. Two different phases were compared, an early phase between 20 and 72 h, and a late phase between 170 and 260 h.

Growth rates of S. quadricauda and C. vulgaris in H_2O based biosludge medium

		Algae specie	
		S. quadricauda	*C. vulgaris*
Early phase	*μ (h^-)[1]*	0.022	0.023
	Generation time (h)	32	30
Late phase	*μ (h^{-1})*	0.011	0.012
	Generation time (h)s	62	60

Samples were dried and the biomass concentration of the two cultures was calculated by dry weight measurements, see Table 11. Final OD_{750} for both cultures were approximately 1.4, but dry weight measurements indicated a large discrepancy between the two cultures. *S. quadricauda* reached 0.95 g/l while *C. vulgaris* only reached 0.28 g/l. These concentrations are lower than those reached on 3N-BBM+V medium, see Table 8. The biomass concentration differences were not visible in OD_{750} measurements, but were clear in the dry weight measurements. This might be because of a complex and problematic background interfering with OD_{750} measurements.

Table 11: Final biomass concentrations of isolated algae species in H_2O-based biosludge medium after 2 weeks of growth. Duplicate samples were measured, the calculated average of each culture is presented.

Final biomass concentration of S. quadricauda and C. vulgaris in H_2O-based biosludge medium

Culture	*Average biomass concentration g/l*	*Maximum deviation*
S. quadricauda	0.95	0.12
C. vulgaris	0.28	0.04

Nutrient concentrations were measured with Hach-Lange cuvette tests and results are presented in Table 12. The acid treated biosludge resulted in higher NO_3 and NH_4 concentrations in the final medium, most notably NO_3 which was almost non-existent in H_2O-treated biosludge medium. Both media were rich in NH_4 and PO_4. The conductive values were translated to % salinity with [72] and resulted in the following:

$$566\ \mu S/cm\ at\ 22\ °C = 0.03\ \%\ Salinity$$ for neutral H_2O-treated biosludge medium

$$17500\ \mu S/cm\ at\ 22\ °C = 1.1\ \%\ Salinity$$ for acidic 1 % HCl-treated biosludge medium

0.03 % salinity is considered fresh water and should not disturb algal growth. 1.1 % is considered brackish.

Table 12: Properties of the two used media for the second screening of isolated species on biosludge-based media. Initial nutrient levels and conductivity were measured as in the previous experiment. Both media show higher nitrogen concentrations than the medium used previously and are also less conductive, indicating a lesser salinity than in prior experiment. 1 % HCl medium contains more nitrogen and phosphorous, but is also 30 times more conductive, indicating a far greater salinity in this medium.

Initial properties and nutrient concentrations for the two different biosludge-based growth media

		H_2O Medium	*1 % HCl Medium*
Nutrients	*NO_3 mg/l*	0.5	32
	NH_4 mg/l	70	90
	PO_4 mg/l	75	86
Conductivity µS/cm		566	17500
Salinity %		0.03 %	1.1 %

Nutrient concentrations in the growth medium were measured after 2 weeks in all cultures, see Table 13. A similar nutrient reduction was found for both growing cultures *C. vulgaris* and *S. quadricauda*. Nutrient concentrations were found to be significantly lower for the growing cultures, compared to cultures showing zero growth, as was expected.

Table 13: Measured growth medium nutrient levels after 2 weeks for growth media used in growth screening 2 of isolated species on biosludge-based media. Nutrients were measured in both growing cultures and one random, non-growing culture each for H_2O- and 1% HCl-based biosludge medium. There were no significant nutrient reduction differences between *C. vulgaris* and *S. quadricauda,* indicating both species has similar nutrient requirements. Nutrient concentrations were significantly higher in cultures showing no growth compared to both *C. vulgaris* and *S. quadricauda.*

Final growth medium nutrient concentrations in both growing cultures in H2O-based medium as well as non-growing cultures with both medium types.

	C. vulgaris	*S. quadricauda*	*non-growing H_2O medium*	*Non growing HCl medium*
NO3 mg/l	0	0	0	30
NH4 mg/l	19	21	67	86
PO4 mg/l	61	56	72	85

4.5. Total lipid content and lipid profile assay of isolated local algal species

A total lipid content and lipid profile assay was performed based on the modified Folch method to evaluate the lipid production for each investigated algae species. The investigated species were *S. dimorphus*, *S. quadricauda*, *C. vulgaris* and *M. contortum*. Algae biomass to be analysed were selected from two performed screenings, algae species cultured in either 3N-BBM+V or treated biosludge-based growth media, see sections 4.4.1 and 4.4.3. Results are visualized in Table 14. All samples were performed and measured in duplicates, with the calculated average lipid content presented in the table. Two samples resulted in significantly higher lipid content than all others, *C. vulgaris* in biosludge medium and *M. contortum* in 3N-BBM+V medium. The calculated total lipid content reached 84 % and 45 % of the dried biomass weight respectively, compared to all others which were approximately 10 – 20 %. The biomass concentration was very low in the *C. vulgaris* sample, 0.28 g/l. this generates a great uncertainty in the calculated lipid content, and caution should be taken in relying on these data alone. No such faults were found for *M. contortum*.

Table 14: The total lipid contents of the investigated algae species are presented below. Algae were selected from two screenings with two different growth media. All samples were performed and measured as duplicates. The lipid content is expressed as a calculated percentage of dry biomass weight. The greatest lipid content were found in *C. vulgaris* cultivated in biosludge medium, and *M. contortum* cultivated in 3N-BBM+V medium. However, *C. vulgaris* resulted in a very low biomass concentration at only 0.28 g/l, which makes the calculated lipid content highly uncertain. All other samples resulted in total lipid contents of 8-19 %.

Total lipid content of algae species cultivated in 3N-BBM+V and treated biosludge medium

Algae species	*Growth medium*	*Biomass concentration (g/l)*	*Total lipid content (%)*
S. dimorphus UTEX 417	3N-BBM+V	1.06	12
S. dimorphus	3N-BBM+V	2.28	19
S. quadricauda	3N-BBM+V	1.88	10
S. quadricauda	Biosludge medium	0.95	8
C. vulgaris	3N-BBM+V	1.04	14
C. vulgaris	**Biosludge medium**	**0.28**	**84**
M. contortum	**3N-BBM+V**	**1.64**	**45**

The lipid profiles of each algae species were evaluated, see Figure 47. Results for *C. vulgaris* cultivated in biosludge medium were omitted due to the uncertain data. Fatty acids from C6 to C24 were measured, with any acids resulting in concentrations above 1 µg/ml visualized in the figure, the rest excluded. The vast majority of the fatty acids were C16:0, C18:2 and C18:1c (cis-form), with traces of C10:0, C16:1, C18:3, C18:0, C20:0 and C24:0 as well. *M. contortum* contained significantly higher concentrations of both C18:2 and C18:1c than all other algae species, with 10 and 23 % w/w respectively. Unsaturated fatty acids are present to a higher extent than saturated fatty acids in *M. contortum*, the other species indicate a more equal distribution among the two.

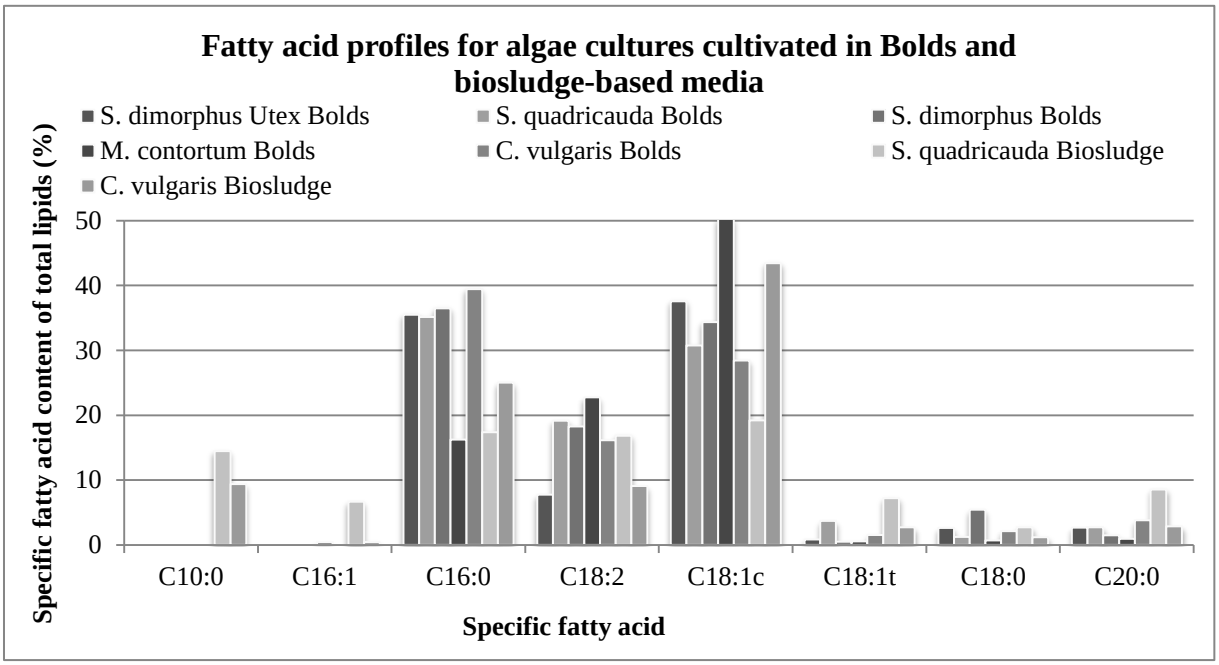


Figure 47: Fatty acid profiles for investigated algae cultures. Specific fatty acids are visualized along the x-axis. The height of the y-axis represents the content percentage of each acid compared to the total lipids for each algae sample. Fatty acids with contents lower than 5 % for all cultures were excluded and the rest are presented in the figure. All samples resulted in similar peaks, with the greatest concentrations of C16:0, C18:2 and C18:1c. *M. contortum* contained the largest fractions of both C18:2 and C18:1c, while *C. vulgaris* displayed the highest concentrations of C16:0.

4.6. CHN-analysis of activated biosludge

Results from a CHN-analysis of the biosludge are presented in Table 15. The nitrogen level were assumed to be *roughly* 2 % w/w based on previous data collected at NPB and was confirmed by this analysis to 2.8 % w/w. Data from NPB showed 2.2 % N w/w which is close to the measured value.

Table 15: The results from the CHN-analysis. *Approximately* 50 % of the total weight was found to be C, H or N. Remaining weight is thought to be mainly oxygen, sulfur and ashes.

CHN-analysis of dried biosludge

Name	Mass	Analysis Date	Carbon %	Hydrogen %	Nitrogen %
Biosludge 1	0.0805	2013-12-11	42.2	6	2.8
Biosludge 2	0.0939	2013-12-11	42.1	5.9	2.8
Average	0.0872		**42.2**	**6.0**	**2.8**

5. Discussion

5.1. Potential use of local isolated algae species from NPB

One aim of this Vinnova and ÅF-funded project was to find suitable species for a slowly stirred raceway-system and Tethys reactors located in Bäckhammar. Local algae species might outgrow any other inoculated algae, thereby minimizing contaminating effects. Seven different algae strains were isolated and stored in pure monocultures in this project. At least ten other species were observed microscopically in the samples, but were never isolated. It is logical to assume the presence of a multitude of algae species within and around the open, non-sterile environment of NPB. However, mixtures of species were also found in the Tethys reactors and raceway system. This indicates difficulties in maintaining sterile cultures throughout the cultivation period. Cultures containing a single alga species are easily defined, but might not present the optimum growth preferences for algae, which might benefit from the presence of bacteria or other algae species. This could result in faster growth rates and thereby, greater productivity [73].

It was not possible to determine the exact species of the isolated algae without performing a genomic investigation, but the observed morphology indicates species within the genera: *Scenedesmus, Monoraphidium, Phormidium, Oscillatoria, Chlorella* and *Coenococcum. Phormidium* and *Oscillatoria are* filament forming algae, while the others are free-living microalgae. Filamentous species might be problematic for biomass production in bioreactors due to unwanted surface fouling and aggregate formation. As light penetration into the bioreactor is crucial for an efficient algae growth fouling is a bad characteristics. On the other hand, filamentous species are easier to separate from the liquid with lower losses due to effective filtration possibilities.

Species of *Scenedesmus, Monoraphidium* and *Chlorella* have all been found interesting in articles regarding growth rates and lipid contents. Bogen *et al* [74] showed an accumulation of 20 % lipids of DW using *Monoraphidium contortum* in 3 l photobioreactors. The lipid assay in this work was performed with cultures in stationary phases. These results indicated a total lipid content of 44 % of the isolated species *M. contortum*, which was the highest lipid content of any evaluated species.

Mandal *et al* [75] used *Scenedesmus obliquus (*a homotypic synonym to *S. dimorphus)* cultured in simulated waste water and was able to show a 9-fold increase in lipid content by nitrogen starvation. Mallick *et al* [76] used *C. vulgaris* and showed it was possible to boost the lipid content to 55 % by nitrogen starvation also. The effect of any nitrogen starvation for these samples is not clear although samples were taken during stationary phases. This might be a beneficial procedure to include in future work to increase lipid yields. Total lipid contents were 19 % for *S. dimorphus* and 14 % for *C. vulgaris in this project*. The lipid profile obtained in this project indicated the necessary fatty acid composition for all evaluated algae species to be suitable for high-quality biodiesel production [77]. The majority of the fatty acids were C16:0, C18:1 and C18:2, which are desirable in biodiesel production.

Macroalgae and filamentous microalgae species are typically rich in carbohydrates and less so in lipid content [78]. Chang *et al* [79] investigated the possibility to use *Phormidium autumnale* for biodiesel production, resulting in 14 % lipid content and the ability to synthesize heptadecane which can be directly used as a biodiesel component without any further transesterification. These filamentous species might be better used as substrates in microbial anaerobic digestion processes instead of direct lipid extraction due to their large fractions of carbohydrates.

5.2. Treated biosludge as a source for nutritious medium

The pilot plant prototype in Bäckhammar aims for using waste materials from the mill to obtain algal biomass. The fertilizer NPK is presently used for supplying nitrogen and phosphorous, but this is not sustainable and an alternative source of N and P must be found. Biosludge was suggested as it contains large amounts of nitrogen (2.8 %). The availability of this nitrogen was investigated by different leaching techniques such as heating, ultrasonification and acid treatment.

Several methods of cultivating algae in media based on biosludge were evaluated. The very first experiment concluded that there was no growth difference for all evaluated treatments in undissolved biosludge. All results were inferior to the two control reactors with NPK and 3N-BBM+V respectively. The following experiment was performed with biosludge dissolved in water, and treated with acid and alkaline methods. Acid sludge treatment resulted in better algal growth than the NPK control reactors after two weeks of growth. Alkaline treatment was also proven to suit algal growth, but might not be feasible in larger scale due to the dark color of the culture medium. Based on these results, acid treatment was chosen as a possible method to optimize nutrient extraction from biosludge.

Screening experiments investigated by algal growth (*S. dimorphus*) were performed to evaluate and optimize the biosludge treatment procedure. Reaction time, temperature and acid strength were tested in various combinations, with results suggesting that nutrient concentrations increased in the culture medium with longer reaction times, higher temperatures and stronger acids. The most effective treatment to release nutrients was using 1 % HCl. However, this medium was problematic in supporting growth of the algae. This may be caused by the addition of acid/base for pH adjustment which increases salt content and hence salinity. Another possibility is the release of metals present in the biosludge at low pH. However, no algal inoculates showed any growth in this medium, nor in the HCl-based medium used in section 4.4.3. Both media contains a high salinity, stemming from the addition of salts with both acid and the base for pH-adjustment, which is problematic when working with freshwater species. One could either perform a high salinity screening to select any local halotolerant algae species, else it is needed to further investigate enhanced nutrient leaching from biosludge with diluted acids or other pre-treatments.

Further, it was hypothesized that metals present in the sludge also were leached into the medium. These metals could interfere with the algal cells and affect their growth negatively [80]. An elemental analysis should be performed to evaluate the metallic composition of the biosludge to investigate which, if any, elements could be leached at toxic concentrations. NPB uses $Al_2(SO_4)_3$ as a precipitate in their waste water treatment process, but this is a substance toxic to algae due to its aluminum-content [81]. The leaching process should be refined to ensure satisfactory nutrient concentrations, while maintaining insignificant concentrations of toxic elements.

5.3. Possible large scale setup at NPB

This project tried to investigate any suitable method of scaling up the results and perform any following experiments *in situ* at NPB. This is one reason why focus has been devoted to developing a simple and inexpensive biosludge treatment method. It is possible to scale up both H_2O- and HCl-based biosludge medium used in screening 2 of isolated species on biosludge-based media for use in cultivation systems at NPB. The medium formulation protocol was constructed for a final volume of 1.5 l, and would be scaled up 1500 x for use in Tethys reactors to reach a working volume 2250 l. This would require 110 kg dried biosludge to be dissolved in 750 l water, and heated by a suitable heat source to 120 °C for 24 h, or lower temperatures for a longer time. The fibers can then be separated from the liquid by sedimentation, in order to replace the filtration step.

Nutrient leaching experiments showed that it was possible to use lower temperatures if one would compensate with longer treatment times. One possible setup would therefore be to prepare the next growth medium alongside an ongoing batch cultivation of algae. The leaching process could commence during the normal 8 month culture season, to be ready at the start of the next year season. Alternatively, if higher temperatures were used a few days would be enough. It would be possible to choose whichever is more suitable since a multitude of possible heat sources can be found at NPB.

Several algae species were found at NPB, but none could survive any of the acid-based biosludge media used in any screening of isolated species on biosludge-based growth media, see sections 4.4.2 and 4.4.3. As discussed earlier one reason could be the high salinity of the medium made by the most effective pre-treatment procedure. However, this could be beneficial at large scale if inoculating a halo-tolerant strain and such cultivation is less prone for contamination since surrounding species are used to fresh water conditions. A suitable inoculate could be *Dunaliella salina. D. salina* is already used in several industrial outdoor cultivations in open ponds for β-carotene production. These open pond systems maintain a high salinity in order to avoid other contaminants [82]. Three of the algae species used in this project, *C. vulgaris*, *M. contortum* and *S. quadricauda*, have been selected for present scaled-up experiments at NPB in 2.3 m^3 Tethys-reactors. These species were selected due to the shown growth rates, which were high in nutritious 3N-BBM+V medium. *C. vulgaris* and *S. quadricauda* also grew in the H_2O-based biosludge medium used in section 4.4.3, indicating promising results if the media fabrication process could be further developed.

6. Conclusion

Many domestic algae species were isolated from NPB and were able to grow in 3N-BBM+V growth medium in the lab. Several cultures were successfully isolated from agar plates and serial dilution as monocultures, indicating robustness to the chosen isolation method.

Biosludge is rich in nitrogen, concluded by an elementary analysis (CHN), but the availability of nitrogen in its pure form is not enough for growing algae. A possible pre-treatment for biosludge has been found, but should be further developed. Nutrient concentrations in the culture medium increased with longer reaction times, higher temperatures and stronger acids. A dilute acid (1 % HCl) together with heat (100 °C) for 5 days proved a great combination pre-treating biosludge, resulting in nutritious medium. The best algal growth was shown in a growth medium based on water instead of HCl, a medium with less nutrients, but also less salts. All tested algae were freshwater species and it is possible that different results would have been observed if marine algae were also investigated. Calculated growth rates were 0.02 h^{-1} for both *C. vulgaris* and *S. quadricauda*, compared to 0.05-0.06 h^{-1} when using 3N-BBM+V medium instead. An elemental analysis focused on metal content should be performed on the biosludge to investigate whether it might cause negative growth effects or not.

Many biosludge pre-treatments were found to yield promising nutrient concentrations in the final growth media. It was found that N-concentrations were limiting compared to the leached P-content, and focus should be to increase the N-concentration. A longer pre-treatment, 4 weeks or more, might be viable in leaching great NO_3^- concentrations and should be conducted since results indicated higher concentrations in longer leaching experiments.

Finally, it could be concluded that it was possible to use waste sources from NPB as a growth medium source for algae growth.

7. Recommendations for future work

- The fastest growth amongst all cultures was observed in a co-culture of *C. vulgaris* and *S. dimorphus* in 3N-BBM+V medium. Future work should evaluate the potential gain in selective co-cultures versus monocultures or open, freely contaminated systems.
- Algae growth on biosludge-based media was observed in experiments with *S. dimorphus UTEX 417* in acid-treated biosludge medium and for *S. quadricauda* and *C. vulgaris* in heat-treated biosludge-based medium. Continued efforts should be made in developing and optimizing a pre-treatment for nutrient leaching in biosludge-based systems. Weaker acid than 1 % HCl should be evaluated, as well as the possibilities to use other acid sources, *e.g.* the flue gas produced *in situ* at NPB.
- Further work should be made at isolating local algae species from NPB. 7 were isolated in this project, but it was obvious that the true amount of available algae species were far greater. Genetic algae strain confirmations should also be performed for any species resulting in promising growth and fatty acid composition.

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8. Appendices

I. Total lipid extraction based on a modified Folch method

Prepare the following working solutions:

A. 2:1 chloroform : MeOH solution

Add 50 ml MeOH to 100 ml chloroform and vortex.

B. 0.73 % NaCl in H2O

Add 730 mg NaCl to 100 ml H_2O and vortex.

C. Hexane solution with 10 µg/ml C17 FAME internal standard.

Add C17 FAME to 200 ml hexane to reach a final concentration of 10 µg/ml.

D. NaMeOH solution

Weigh 1.2 g pure Na and add to 100 ml MeOH. Keep the container open and let the solution react fully before capping the container and vortexing the solution.

Lipid extraction protocol:

1. Transfer 1 ml wet alga biomass suspension to 1.5 ml Eppendorf tubes.
2. Centrifuge 5 min at 14000 rpm and discard the supernatant.
3. Add 200 µl of solution A to all tubes, together with 10 µl C19 IS and vortex.
4. Treat in ultra-sonic bath for 60 min.
5. Centrifuge 5 min at 14000 rpm and transfer 150 µl of the lower oil phase to new tubes.
6. Wash the pellet with 200 µl of solution A and vortex.
7. Treat in ultra-sonic bath for 15 min.
8. Centrifuge 1 min at 14000 rpm and transfer 150 µl of the lower oil phase to the same tubes as in step 5.
9. Repeat steps 6-8 3 times.
10. Add 160 µl of solution B to the new tubes and vortex.
11. Centrifuge 3 min at 14000 rpm and remove the top phase. Let remaining oil phase dry by evaporation over night.
12. Resolve samples by adding 200 µl of solution C and vortex.
13. Treat in ultra-sonic bath 10 min.
14. Repeat step 12-13 3 times.
15. Add 400 µl solution D to the tubes and let react over night.
16. Add 200 µl MQ H_2O to all tubes and vortex.
17. Centrifuge 5 min 14000 rpm and remove the lower polar phase.
18. Repeat step 16-17 3 times.
19. Transfer top oil phase to crimped GC-MS vials for analysis.

II. Biosludge and NPK calculations based on nitrogen levels

Amount of biosludge and NPK used in the nutrient leaching screenings were based on the premise to achieve similar nitrogen levels in all reactors. Bolds medium was used as a benchmark for total available nitrogen.

Total nitrogen in 5l 3N-BBM+V

$NaNO_3$ in 1l 3N-BBM+V:

$$25\ g\ NaNO_3 * 0.030\ L = 0.75\ g$$

N % in $NaNO_3$:

$$\%\ N = \frac{M(N)}{M(NaNO_3)} = \frac{14.0067}{84.99467} = 16.480\ \%$$

N in 1l 3N-BBM+V:

$$m(N) = m(NaNO_3) * N\% = 0.75\ g * 16.480\% = 0.1236\ g = 123.6\ mg$$

N_{tot} in 5l 3N-BBM+V medium:

$$N_{tot} = 123.6\ mg/l * 5\ l = 618\ mg$$

Total available nitrogen: 618 mg

Biosludge needed for similar nitrogen availability

N % in biosludge:

$$N\ in\ biosludge = 2.2\%\ (Data\ from\ NPB)$$
$$N\ in\ biosludge = 2.8\ \%\ (Data\ from\ elemental\ analysis\ at\ SP, Borås)$$

Biosludge needed:

$$m(biosludge) = \frac{Total\ available\ nitrogen}{average\ N\%} = \frac{618\ mg}{2.5\%} = 24.7\ g\ \cong 25\ g$$

Total biosludge needed: 25 g biosludge in 5l deionized water

NPK needed for similar nitrogen availability

N % in NPK:

$$N\ in\ NPK = 14\ \%$$

NPK needed:

$$m(NPK) = \frac{Total\ available\ nitrogen}{N\%\ in\ NPK} = \frac{618\ mg}{14\ \%} = 4.41\ g$$

Total NPK needed: 4.43 g in 5L deionized water

III. Recipe 3N-BBM+V (Bolds Basal Medium, 3-fold Nitrogen + Vitamins)

Stocks	**per liter**	
(1) $NaNO_3$	-	25.0 g
(2) $MgSO_4.7H2O$	-	7.5 g
(3) NaCl	-	0.5 g
(4) $K_2HPO_4.3H2O$	-	7.5 g
(5) KH_2PO_4	-	17.5 g
(6) $CaCl_2.2H2O$	-	2.5 g

(7) Trace elements solution

Add to 1 liter of distilled water 0.75 g Na_2EDTA and the minerals below in exactly the following sequence:

$FeCl_3.6H2O$ - 97.0 mg
$MnCl_2.4H2O$ - 41.0 mg
$ZnCl_2.6H2O$ - 5.0 mg
$CoCl_2.6H2O$ - 2.0 mg
$Na_2Mo_4.2H2O$ - 4.0 mg

(8) Vitamin B1

0.12 g Thiaminhydrochloride in 100 ml distilled water. Filter sterile.

(9) Vitamin B12

0.1 g Cyanocobalamin in 100 mg distilled water, take 1 ml of this solution and add 99 ml distilled water. Filter sterile.

Medium

Stock solution **1** - 30.0 ml
Stock solutions **2** - **6**, 10.0 ml each

Stock solution **7** - 6.0 ml
Stock solutions **8** - **9**, 1.0 ml each

IV. NPK medium formulation method

1. Calculate desired final concentration of NPK. For example calculations of desired concentrations used in this work, see Appendix II.
2. Weigh correct amount of NPK fertilizer.
3. Dissolve in deionized water.
4. Sterilize by autoclaving at 120 °C for 15 min.

V. Nutrient measurements with Hach-Lange cuvette test kits.

-- NO_3^- --

1. Add 0.2 ml sample to a cuvette.
2. Add 1 ml reagent A.
3. Shake and mix.
4. Let rest for 15 min.
5. Measure absorbance at 370 nm.
6. Translate absorbance to concentration with standard curves (see Appendix VI.)

-- NH_4^+ --

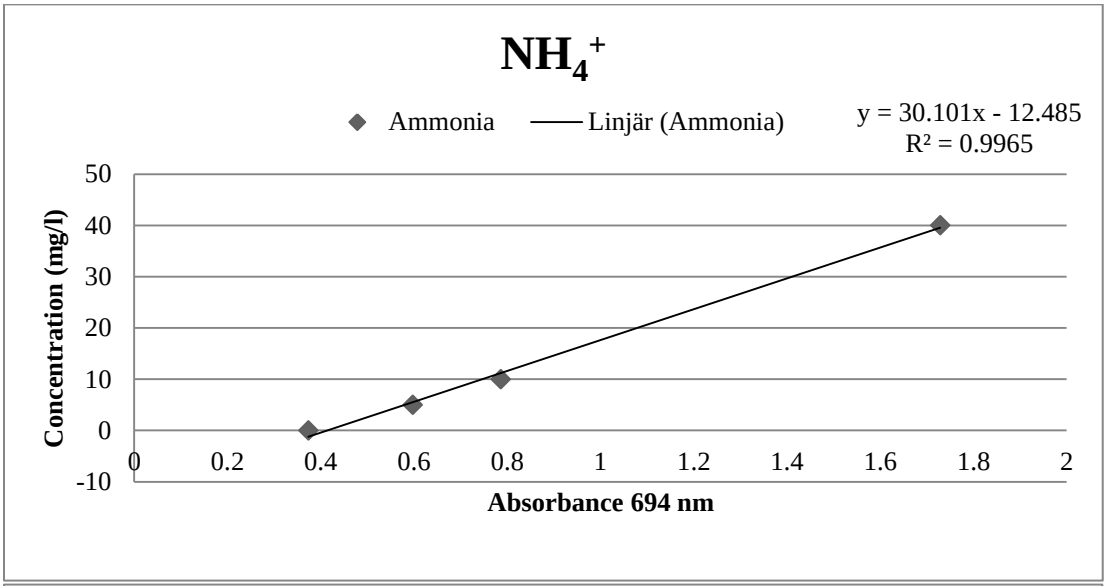
1. Add 0.2 ml sample to a cuvette.
2. Remove film to expose reagent in cap.
3. Replace cap and shake mixture
4. Let rest for 15 min.
5. Measure absorbance at 694 nm.
6. Translate absorbance to concentration with standard curves (see Appendix VI.)

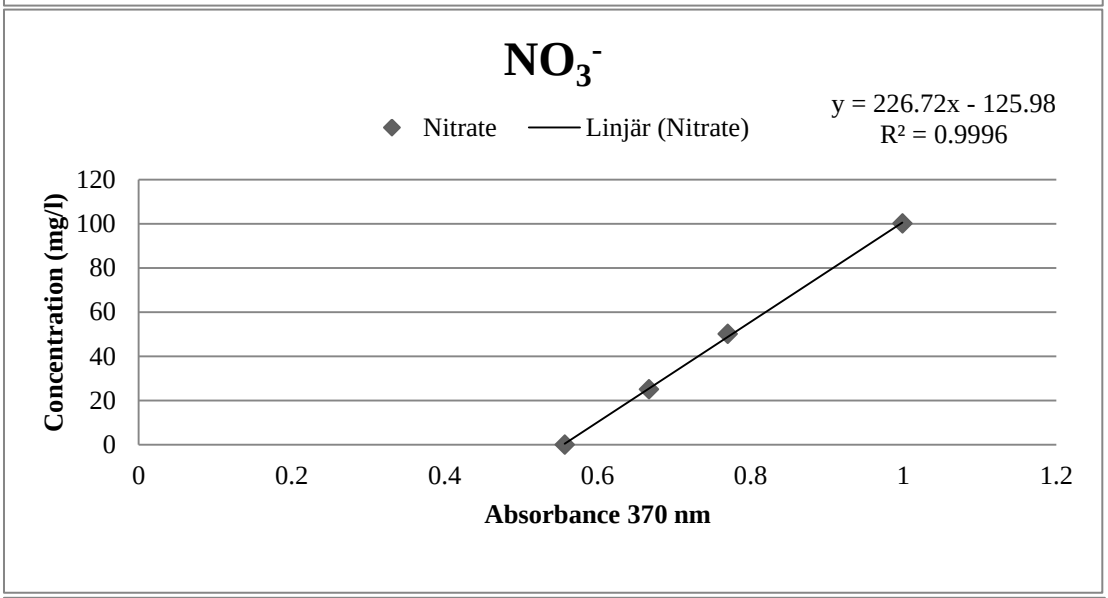
-- PO_4^{3-} --

1. Add 0.4 ml sample to a cuvette
2. Add 0.5 ml reagent B.
3. Replace cap with cap C.
4. Shake and mix.
5. Let rest for 10 min.
6. Measure absorbance at 750 nm.
7. Translate absorbance to concentration with standard curves (see Appendix VI.)

VI. Standard curves and calculations for NH4+, NO3- and PO43-

The constructed standard curves below were used to translate measured OD-values to concentrations





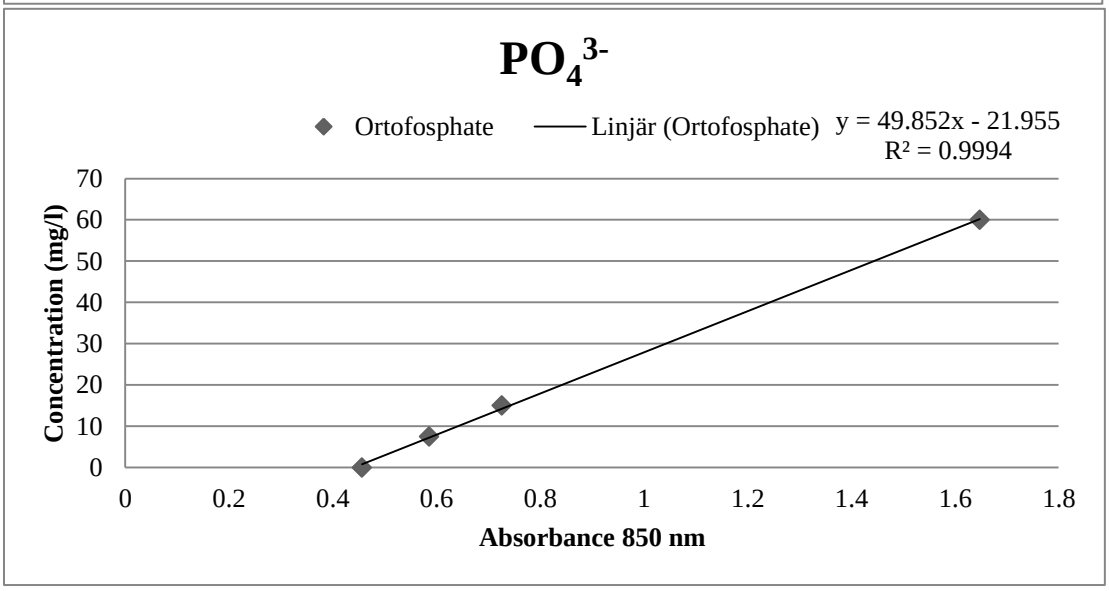


Table 16 shows measured absorbance for each separate run with varying time and temp parameters. These numbers were averaged and used together with the standard curves to form concentration curves instead.

Table 16. Measured absorbance in triplicate samples from the nutrient leaching experiment. Each combination of temperature and time is expressed in bold after each run number, *e.g.* Run 1 combined 25 °C with 1 h time. The resulting absorbance was noted in each column corresponding to the correct nutrient.

Run 1	***25,1***		***Run 2***	***25,24***		***Run 3***	***25,48***	
NH4	*NO3*	*PO4*	*NH4*	*NO3*	*PO4*	*NH4*	*NO3*	*PO4*
0.4873	0.6097	0.6404	0.4664	0.6725	0.983	0.5573	0.7842	0.9501
0.4577	0.5872	NaN	0.502	0.6293	0.9722	0.4981	0.8027	1.0556
0.4581	0.5432	0.9283	0.4776	0.6584	0.9279	0.537	0.8084	1.1986
Run 4	***70,1***		***Run 5***	***70,24***		***Run***		
NH4	*NO3*	*PO4*	*NH4*	*NO3*	*PO4*			
0.545	0.5464	1.2167	0.8355	0.7232	1.6163			
0.4634	0.5889	1.8182	0.7936	0.6777	1.5653			
0.5183	0.6237	0.565	0.8717	0.6113	1.6538			
Run 6	***120,1***		***Run 7***	***120,24***		***Run***		
NH4	*NO3*	*PO4*	*NH4*	*NO3*	*PO4*			
0.8527	0.5633	1.5572	1.1194	0.691	1.722			
0.9026	0.6196	1.6052	1.032	0.7597	1.6262			
0.9172	0.6155	1.5937	1.0135	0.723	1.6057			

Example calculations for Run 1, 25 °C and 1 hour:

$$Conc_{NH_4^+} = \bar{x}(0.4873, 0.4577, 0.4581) * 30.101 - 12.485 = 0.4677 * 30.101 - 12.485$$
$$= 1.59 \frac{mg}{l}$$

$$Conc_{NO_3^-} = \bar{x}(0.6097, 0.5872, 0.5432) * 226.72 - 125.98 = 0.5800 * 226.72 - 125.98$$
$$= 5.53 \frac{mg}{l}$$

$$Conc_{PO_4^{3-}} = \bar{x}(0.6404, 0.9283) * 49.852 - 21.955 = 0.7844 * 49.852 - 21.955$$
$$= 17.15 \frac{mg}{l}$$

VII. Growth rate and generation time calculations

Example calculations for specific growth rate and generation time for acid treated biosludge from nutrient leaching 2.

$$Specific\ growth\ rate = \frac{ln(Y_2) - ln(Y_1)}{(X_2 - X_1)}$$

Where y denotes measured OD-values and X denotes time between measure points 1 and 2.

$$Generation\ time = \frac{\ln(2)}{Specific\ growth\ rate}$$

Specific growth rate and generation time for early phase acid biosludge:

$$Specific\ growth\ rate_{early\ phase\ acid} = \frac{ln\left(\frac{0.16165 + 0.1066}{2}\right) - ln\left(\frac{0.1635 + 0.0227}{2}\right)}{(94.75 - 0)}$$
$$= 0.02034\ d^{-1}$$

Where *y(1)* and *y(2)* where averaged for the two reactors.

$$Generation\ time = \frac{\ln(2)}{0.02034} = 34.08\ h$$

VIII. Biosludge medium formulation method

1. Dry biosludge at 80 °C until completely dry.
2. Weigh 50 g dry biosludge and place in container.
3. Prepare 500 ml working solution of 5 M NaOH by dissolving 100.0 g NaOH in 500 ml deionized water.
4. Dilute 250 ml fuming 37 % HCl with 500 ml deionized water.
5. Add 250 ml deionized water to the biosludge.
6. Add a stirring magnet and place on stirrer. Stir rapidly until fibers are more or less dissolved.
7. Remove magnetic stirrer and add acid. The final concentration is 10 % HCL in deionized water.
8. Cover with aluminium foil and keep undisturbed for 1 week, alternatively measure nutrient levels and use when nutrients have reached suitable levels.
9. Filter solution through a Munktell 3 filter and discard remaining fibers.
10. Collect the filtrate and adjust to pH 7 by addition of NaOH working solution. OBS! When adjusting pH the solution will darken and form salts.
11. Let salts sediment and remove the clear phase of the liquid.
12. Filter remaining liquid through a Munktell 00A filter and collect the filtrate. Add to previously collected liquid.

IX. Method for growth screenings 1 and 2 of *S. dimorphus* on treated biosludge

1. Autoclave all equipment used in the screening at 120 °C for 15 min.
2. Dry biosludge in 80 °C until completely dry
3. Weigh, net and pre-treat biosludge for each experiment.
4. Prepare nutritious control media of NPK and 3N-BBM+V.
5. Calculate appropriate dilutions of algal pre cultures to the final volume used in screening.
6. Centrifuge calculated volumes of algal pre culture at 1100 xg for 5 min
7. Gently discard the supernatant. Try not to disturb the pellet as it is soft.
8. Resuspend pellet in sterile water. Add the same volume that was discarded.
9. Add biosludge and control media to reactors.
10. Add algal pre cultures to reactors.
11. Initiate stirring and gas flow. Inspect and if necessary, manually adjust for an even flow across all reactors. Set the gas flow to 1 l/min

X. Method of nutrient leaching screening

1. Wash and dry PTFE-chambers
2. Dry biosludge in 80 °C until completely dry.
3. Add 0.5 g biosludge to PTFE-chamber.
4. Prepare 10% working solution of HCl. Dilute concentrated HCl with sterile water.
5. Add 50,0 ml 10% HCl to PTFE-chambers and seal them tight. Mix the contents by gently shaking the chamber.
6. Place PTFE-chambers in appropriate temperature and leave them until time to measure.
7. When it is time for measurements, let chambers cool to room temperature.
8. Filter acid and sludge through a Munktell 3 filter to remove fibers. Discard filter and remaingin fibers and collect filtrate.
9. Adjust pH by addition of 2 M NaOH to pH 7.
10. Measure nutrient levels with correct measure kit.

XI. Method for growth rate screenings of isolated algae species

1. Autoclave all equipment used in the screening at 120 °C for 15 min
2. Prepare nutrition medium (see appropriate Appendix)
3. Measure pH of medium. Adjust if necessary to pH 7.
4. Calculate appropriate dilutions of algal pre cultures to the final volume used in screening.
5. Add calculated volume of nutritious medium to reactors.

   -- If cultures are kept in the same medium used in the screening, skip to step 9 --

6. Centrifuge calculated volumes of algal pre culture at 1100 g for 5 min
7. Gently discard the supernatant. Try not to disturb the pellet as it is soft.
8. Resuspend pellet in the nutritious medium used in the screening. Add the same volume that was discarded.
9. Add calculated volume of algal pre culture to reactors.
10. Initiate gas flow via the program gas mixer.exe. Start with a total flow at 1.4 l/min and 0 % CO_2
11. Immediately measure pH and OD_{750} to verify calculations and yield an initial OD_{750} for growth rate calculations.
12. Adjust gas flow according to Figure 19 and measure pH and OD_{750} continually.